



PHD

Modulation of root antioxidant status to delay cassava post-harvest physiological deterioration

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Modulation of Root Antioxidant Status to Delay Cassava Post-Harvest Physiological Deterioration

Copy 1 of 3

Michael Timothy Page

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

May 2009

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Michael T. Page

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ABBREVIATIONS

$^1\text{O}_2$	singlet oxygen
ABA	abscisic acid
AFLP	amplified fragment length polymorphism
APX	ascorbate peroxidase
BAP	6-benzylamino purine
CAT	catalase
CIAT	Centro Internacional de Agricultura Tropical
CMD	cassava mosaic disease
DAF	days after fertilisation
DAG	days after germination
DAP	days after planting
DW	dry weight
FEC	friable embryogenic callus
FW	fresh weight
GuPX	guaiacol peroxidase
GusP	GusPlus
H_2O_2	hydrogen peroxide
HNL	hydroxynitrile lyase
HPX	horseradish peroxidase
HR	hypersensitive response
IITA	International Institute of Tropical Agriculture
LA	linolenic acid
LAH	lipolytic acyl hydrolase
LAI	leaf area index
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
MAS	marker assisted selection
MeJa	methyl jasmonate
METC	mitochondrial electron transport chain
MV	methyl viologen
NAA	naphthalene acetic acid
NFW	nuclease free water
O_2^-	superoxide radical
OH^\bullet	hydroxyl radical
PAL	phenylalanine ammonia-lyase
PCD	programmed cell death
PLP	patatin-like protein
POX	peroxidase
PPD	post-harvest physiological deterioration
RH	relative humidity
ROS	reactive oxygen species
SDW	sterile distilled water
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SOD	superoxide dismutase
sqPCR	semi-quantitative PCR
UTR	untranslated region
WDM	Weeder derived motif
WT	wild-type

ABSTRACT

Cassava ranks seventh in terms of worldwide crop production, providing a staple for over half a billion people. The production of cassava is limited by several factors, with post-harvest physiological deterioration (PPD) of storage roots a major constraint. PPD is a process initiated on harvesting and mediated by reactive oxygen species (ROS) that ultimately renders storage roots unpalatable and unmarketable. It is similar to a conventional plant wound response, but crucially lacks efficient wound repair and down-regulation of stress signalling. Therefore, the strategy utilised here to modulate PPD focussed on increasing the ROS scavenging ability of storage root tissue through a biotechnological approach.

Three expression plasmids were produced, harbouring cassava genes encoding the antioxidant enzymes APX, CAT and SOD under the control of the storage root-specific *StPAT* promoter. In addition, a reporter expression plasmid was created, with *StPAT* driving the expression of *GusP*. Transgenic *Arabidopsis* plants containing the *StPAT::GusP* cassette demonstrated root-specific GusP staining. Non-root tissue also showed wound-inducible GusP activity conferred by the *StPAT* promoter. This novel activity was detected almost immediately after wounding and occurred independently of ethylene, MeJa and ROS. The 3' 261 bp of the *StPAT* promoter was sufficient to confer wound-inducible expression and contained putative wound responsive *cis* regulatory motifs. Analysis of PATATIN function indicated a role during early responses to wounding in the liberation of free fatty acids from cell membranes.

Over-expression of the target genes in the model plant *Arabidopsis* increased the antioxidant enzyme activity in the roots of selected lines. Transgenic plants generally exhibited similar levels of oxidative stress resistance to wild-type plants, a result due in part to the efficient nature of the oxidative stress response of *Arabidopsis* – the APX activity of wild-type plants increased to transgenic levels under H₂O₂ stress. However, PPD in cassava is at least partially the result of a poor antioxidant response to harvesting, and so transformation of cassava with the expression plasmids remained a viable strategy. Transgenic cassava plants harbouring the expression cassettes are being generated and will soon be assessed for PPD resistance.

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1 INTRODUCTION

1.1 Cassava – Taxonomy, Origins & Distribution

Cassava (*Manihot esculenta* Crantz), also known as tapioca, yuca and manioc, is a dicotyledonous, diploid ($2n=36$), perennial shrub belonging to the Euphorbiaceae, a group that includes agronomically important species such as rubber (*Hevea brasiliensis*) and castor bean (*Ricinus communis*). The Euphorbiaceae group is distinguished by the ability to produce latex, although cassava is not widely grown for this purpose (Reilly, 2001).

Cassava's geographical origin has long been debated, although it is now thought to derive from a major centre of diversity of the 98 *Manihot* species in Central Brazil (Allem, 2002; Nassar, 2002). From a botanical standpoint, it was assumed until relatively recently that cassava had no known ancestry. The discovery of wild populations of cassava altered this supposition and led to the reclassification of three subspecies; *Manihot esculenta* Crantz ssp. *esculenta* (cultivated), *Manihot esculenta* Crantz ssp. *flabellifolia* (wild) and *Manihot esculenta* Crantz ssp. *peruviana* (wild) (Allem, 2002). These two wild progenitor subspecies, along with the closest wild relative of cassava *Manihot pruinosa*, form the primary gene pool of the crop (Allem, 2002).

The agricultural origin of cassava, that is the first site of domestication between 6,000 and 8,000 years ago, is also highly contested and numerous sites in South America have been suggested (Allem, 2002). A phylogeographic approach, assessing polymorphisms within the single-copy nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*), identified the South Amazon border with Bolivia as a likely origin of domestication (Olsen & Schaal, 1999). The introduction of cassava to Africa and Asia by the Spanish and Portuguese occurred during the sixteenth and seventeenth centuries respectively (Reilly, 2001).

Today cassava is cultivated for its starchy storage roots in tropical and subtropical regions between 30 °N and 30 °S. Africa is the world's largest producer of cassava (53.94%), with Asia (29.61%), Latin America (16.36%) and Oceania (0.09%) also contributing (FAOSTAT, 2009). In Africa, where dependency on cassava is greatest, the major cassava producers lie in a band stretching from west to east between 10 °N and 20 °S (Figure 1.1). The crop

requires a mean daily temperature above 20 °C for optimum growth, although it is grown in high altitude tropical areas where the climate is cooler and therefore growth is slower (El-Sharkawy, 2004).

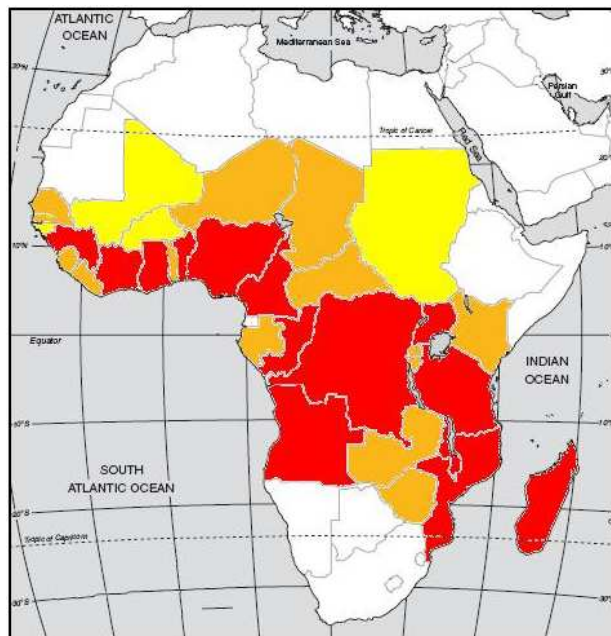


Figure 1.1: Cassava production in Africa. Red $\geq 1,000,000$ tonnes, orange $\geq 100,000$ tonnes, yellow $< 100,000$ tonnes, white = no production (FAOSTAT, 2009).

1.2 Cassava Growth

Cassava is able to grow on nutrient-poor soils and can tolerate long periods of drought. As a result, it is often grown on marginal soils unsuitable for the cultivation of other crops. Cassava is a perennial shrub that commonly grows up to four metres in height (Alves, 2002), although this is influenced by climate and cultivar. The growth cycle of cassava does not always follow a strict regime as environmental factors, such as the effect of seasons, play significant roles in dictating growth characteristics and so a general cassava growth cycle is described here.

The crop is propagated via hardwood stem cuttings that contain sufficient carbohydrate and mineral reserves needed for the initial growth of roots and leaves (El-Sharkawy, 2004). These 'stakes' form adventitious roots from the basal

surface at seven days after planting (DAP). By 15 DAP leaf emergence has usually occurred although true leaves appear only after 30 DAP. At this point fibrous roots also start to develop and will penetrate the soil to a depth of 40-50 cm, functioning in water and nutrient uptake. From 60 to 90 DAP, storage roots become distinguishable from the fibrous roots, continuing to bulk up to 300 DAP (El-Sharkawy, 2004). The highest rate of vegetative growth is seen between 90-180 DAP, during which time the plant architecture is also established. From 180-300 DAP, root bulking accelerates, leaves begin to senesce and the stem becomes lignified. After 300 DAP, plants may experience a period of inactive growth, after which vegetative growth and storage root bulking can resume (Alves, 2002).

1.3 Cassava Storage Root Physiology

There are numerous cassava cultivars with diverse morphological features. Cultivars may be distinguishable by storage root characteristics such as number, bulking time or diameter. Given this intrinsic variability, the information given below is a general description of the physiology of cassava storage roots.

The edible cassava storage root is derived entirely from root tissue and therefore cannot be considered a true tuber as these are derived from stem tissue. Indeed, the secondary thickening of fibrous roots that leads to storage root formation may be an abnormal but advantageous phenotype selected for by man since domestication of the species. As a consequence of this, the storage roots cannot be used as propagules as they can in true tuber crops since the formation of bud primordia does not occur, and it is for this reason that propagation is performed via woody stem cuttings. This method of propagation limits the multiplication rate of the crop, although it does ensure the economically important part of the crop does not have to be reinvested in the production of new planting material and so can be fully utilised (Cock, 1985).

The cassava storage root is comprised of three distinct tissues: bark (periderm), peel and the edible parenchyma (Figure 1.2). The periderm (3% of the total weight) is a thin layer and is partially sloughed off during storage root growth. The peel constitutes 11-20% of the root (fresh weight, FW) with the remainder (77-86%) accounted for by the edible parenchyma (Alves, 2002). The parenchyma colour varies greatly between cultivar and is positively correlated with the total carotenoid content of peeled storage roots (Sánchez *et al.*, 2006). Cassava

storage roots are primarily cultivated for their high carbohydrate content, which may lie between 85-91% of the total dry weight (DW) (Alves, 2002). However, as well as providing a small amount of protein (1.76-2.68% FW, 0.77-8.31% DW) (Alves, 2002; Chávez *et al.*, 2005), cassava storage roots are a rich source of vitamins and minerals (Table 1.1). Indeed, a daily consumption of just 125 g would provide 100% of the dietary intake of ascorbate (vitamin C) for an adult male.

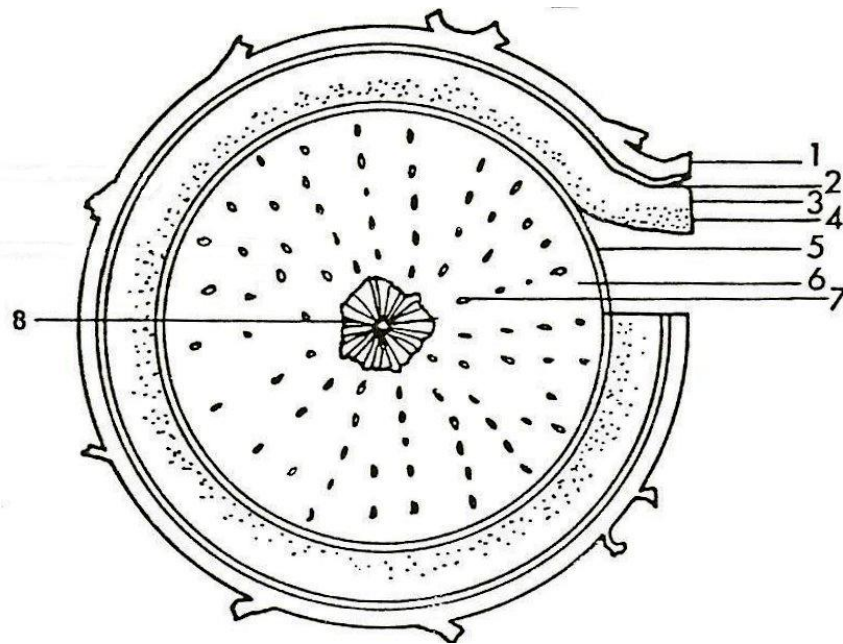


Figure 1.2: Transverse section of a cassava storage root. 1 = bark/periderm, 2 = sclerenchyma, 3 = cortical parenchyma, 4 = phloem, 2-4 = peel, 5 = cambium, 6 = storage parenchyma, 7 = xylem vessel, 8 = xylem vessels and fibres, 5-8 edible parenchyma. Reproduced from Hunt *et al* (1977).

Table 1.1: Nutritional properties of cassava storage roots

Nutrient	Range in mg kg ⁻¹ (mean)	Guideline daily intake mg/day ^a	Reference
iron	6.0 – 230.0 (17.1)	9.1 – 27.4 ^b	Chávez <i>et al.</i> , 2005
magnesium	806 – 1,479 (1,153)	260	Chávez <i>et al.</i> , 2000
zinc	2.6 – 37.5 (7.5)	4.2 – 14 ^b	Chávez <i>et al.</i> , 2005
ascorbate	(360)	45.0	Cock, 1985
thiamine	(0.6)	1.2	Cock, 1985
riboflavin	(0.3)	1.3	Cock, 1985
niacin	(6)	16	Cock, 1985

a = based on data for an adult male (WHO & FAO, 2004).

b = dependent on bioavailability.

An individual cassava plant may possess 3-15 storage roots per plant, with the total yield not exceeding 3.4 kg (FW) (Alves, 2002; El-Sharkawy, 2004). Once a fibrous root has begun to develop into a storage root, its ability to absorb water and nutrients is substantially reduced (Alves, 2002). It is thought that storage root formation is initiated when the level of photoassimilates fixed by the leaves exceeds that required for leaf and stem growth (Lian & Cock, 1979). Leaf area index, the ratio of the area of the upper surface of all the leaves on a plant to the area of ground that the plant covers also correlates with the rate of storage root bulking, further supporting this theory (Cock *et al.*, 1979). These insights demonstrate how cassava storage roots function as sinks for a plant's nutritional resources. These resources remain partitioned until resource import is limited, for example during periods of drought when a large proportion of a plant's leaves may be shed.

1.4 Cassava as a crop

1.4.1 The potential of cassava

Cassava is the world's seventh most important food crop in order of production - world production of cassava in 2007 was 2.28×10^8 tonnes, which equates to over 30% of all tuber crops (FAOSTAT, 2009). The starchy storage roots provide the staple source of carbohydrates for over half a billion people (El-Sharkawy, 2004; Nassar & Collevatti, 2005) and 37% of the total calorific intake in Africa (Rickard &

Coursey, 1981). They are a source of minerals and vitamins but have a relatively low protein content compared to other tuberous crops. The storage roots are also used to produce animal feed and industrial products such as paper and textiles. Cassava is propagated via woody stem cuttings enabling all harvested storage roots to be used as food. It is estimated that cassava is achieving 15% of its production potential (Fauquet & Taylor, 2002), thereby implying that improved growing material coupled with improved agronomic practice could have a major impact on the future production of this crop.

Cassava is regularly grown on marginal land as it is able to grow and produce storage roots in soil of poor nutritional value. This makes it an attractive crop for farmers in the tropics since land unsuitable for other agronomic practices can be utilised. It also enables cassava to be used as part of an intercropping strategy since it can be grown at the end of a cropping cycle when the soil quality is at its lowest.

Cassava serves as a famine-reserve crop in the tropics as it is able to tolerate periods of drought as long as 6 months (Lokko *et al.*, 2007). While the storage roots act as a source of energy to aid recovery of the plant after periods of drought, several other mechanisms of water retention and water stress avoidance play a role in drought tolerance in cassava. Rapid stomatal closure during the middle of the day when temperatures are highest prevents water loss through transpiration. The opening of stomata in the morning and late afternoon ensures that transpirational water loss only occurs during periods of high crop water use efficiency (Connor & Palta, 1981; El-Sharkawy, 2004). Leaf drooping during the hottest part of the day also reduces transpirational water loss by decreasing leaf light interception and thereby reducing the photosynthetic rate. A slight reduction in turgor pressure in the pulvinus at the base of cassava leaves is sufficient to bring about leaf drooping (Alves, 2008). During severe drought, cassava will shed leaves while maintaining a reduced rate of photosynthesis in the remaining leaves, ensuring available soil water is used slowly (El-Sharkawy, 2004). Other mechanisms of drought tolerance in cassava include deep rooting to reach available soil water (Connor *et al.*, 1981) and minimising leaf growth until the pressure of drought stress is relaxed (Alves & Setter, 2004).

1.4.2 PPD – A major constraint on cassava production

As previously stated, cassava is said to be achieving 15% of its production potential (Fauquet & Taylor, 2002). Cassava did not reap the benefits of the Green Revolution that drastically improved yields of wheat, maize and rice. Consequently it possesses several unfavourable characteristics that have not been improved by conventional breeding or biotechnology.

There are numerous constraints on cassava production. Several of these are common to all tuber crops, such as invertebrate attack, viral infection and microbial decay while others, such as the production of cyanogens, are specific to cassava. Cassava storage roots, unlike all other crop tubers, suffer from a remarkably short shelf-life due to a physiological deterioration that renders them unpalatable and unmarketable within three days. This process, known as post-harvest physiological deterioration (PPD), is visible as a blue-black discolouration of the vascular tissue (Figure 1.3) and an associated increase in fluorescence under UV light. It is temporally distinct from microbial decay, occurring before bacterial or fungal attack (Reilly *et al.*, 2004).

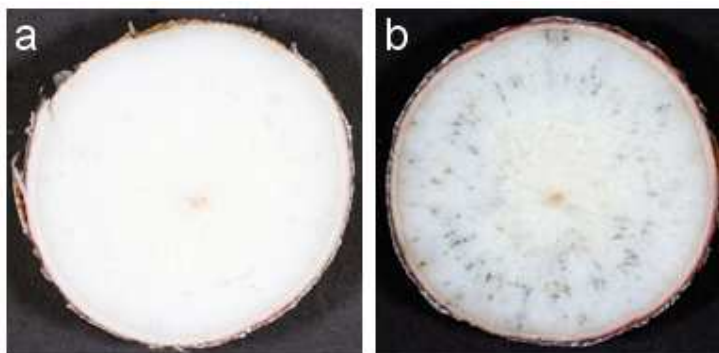


Figure 1.3: Discolouration of the vascular tissue is used to assess the extent of PPD. Shop bought cassava storage roots (a) on harvesting and (b) 48 hours later. Photographs taken by John Beeching.

Traditionally PPD has not had a significant impact on cassava production as storage roots may be left in the ground until needed and then processed or consumed immediately. However, as population sizes and the level of urbanisation in developing tropical countries are increasing, market places are becoming more centralised. Consequently, there is a necessity to transport

storage roots from the field to the market place, increasing the time between producer and consumer (Reilly *et al.*, 2004). As a result, PPD is evolving into a major constraint on global cassava production.

Estimates of cassava losses due to PPD are rarely accurate and depend highly on variety, climate and distance between producer and consumer. That said, post-harvest losses of cassava could be as high as 30% (Wenham, 1995), where PPD is the major cause. PPD also results in market price reductions on old roots and price mark-ups on fresh roots. Ultimately this encourages consumers to choose alternative supplies of carbohydrates, increasing dependency on imported food.

The enhancement of the shelf-life of cassava storage roots from a few days to two weeks could resolve 90% of the deterioration constraints associated with the crop and enhance its potential as an economic commodity (Wenham, 1995).

1.5 PPD – biochemical and molecular mechanisms

1.5.1 Early biochemical research on PPD

Given its use in visually diagnosing PPD, it is understandable that blue-black vascular streaking received much of the focus concerning research on the nature of PPD. Vascular streaking in cassava is characterised by the presence of an occluding compound and tyloses in the xylem channel (Rickard & Marriott, 1979). PPD was first identified as enzymatic after the inhibition of the process by heating to 53 °C for 45 minutes (Averre, 1967). It is now generally accepted that streaking is caused by peroxidase-mediated oxidation of the coumarin scopoletin in the presence of hydrogen peroxide, which gives a dark blue product of unknown structure (Wheatley & Schwabe, 1985; Reilly *et al.*, 2004). This theory is supported by: the presence of peroxidase isoforms with activity towards scopoletin in cassava storage roots (Reilly *et al.*, 2004); the abundance of hydrogen peroxide in deteriorating roots; and the up-regulation of peroxidase expression in roots during PPD (Rickard & Gahan, 1983). Furthermore, scopoletin fluoresces bright blue under UV light and an increase in fluorescence is the earliest visible symptom observed during deterioration (Wheatley & Schwabe, 1985). Additionally, scopoletin is synthesised via the phenylpropanoid pathway and phenylalanine ammonia-lyase (PAL), a key enzyme in this pathway, is up-regulated after harvesting in cassava storage roots (Rickard, 1981). The involvement of scopoletin in vascular discolouration is further supported by the evidence that

application of exogenous scopoletin to cassava root cubes gave on average over 65-fold more discoloured vessels than a water treatment, with a range of other phenolic compounds showing little difference from the water control (Wheatley & Schwabe, 1985). As well as scopoletin, the fluorescent coumarins esculin and scopolin also accumulate in the storage root after harvesting. Scopoletin levels peak first after 20 hours, with esculin and scopolin peaking after 40 hours (Tanaka *et al.*, 1983). PPD has also been demonstrated to be associated with ethylene biosynthesis and increases in the respiration rate of storage roots (Hirose *et al.*, 1984).

Early research also demonstrated conclusively that microbes were not the causal agents of PPD. The inability to isolate micro-organisms from discoloured tissue up to 7 days post-harvest (Averre, 1967; Booth, 1976) and the failure of artificial infections to generate symptoms akin to PPD (Noon & Booth, 1977) proved that PPD was mutually exclusive from microbial decay. The treatment of storage roots with fungicides (up to 20,000 ppm dicloran and benomyl) and bactericides (2.5% streptomycin sulphate) (Averre, 1967; Noon & Booth, 1977) could not prevent PPD, extending the evidence for the absence of microbes during the process.

It is now accepted that PPD is an active process. The treatment of cassava root discs with cycloheximide, an inhibitor of protein synthesis, reduced UV fluorescence in a dose-dependent manner (Uritani *et al.*, 1984) proving PPD is associated with the production of new protein. Further supporting this theory, the *in vivo* labelling of proteins with radioactive amino acids demonstrated a substantial increase in radioactive protein after harvesting indicating a large induction of protein synthesis (Beeching *et al.*, 1994). Using this method, cassava storage roots were shown to be essentially translationally dormant on harvesting, remaining so for up to 8 hours afterwards. Between 8 and 24 hours post-harvest, *de novo* protein synthesis escalated, remaining high to at least 48 hours post-harvest.

1.5.2 Reactive oxygen species – production, scavenging and function

Given that the exclusion of oxygen from storage root wound sites delayed PPD and that there is an ingress of oxygen into the root after harvesting, it was hypothesised that reactive forms of oxygen (reactive oxygen species, ROS) could play a crucial role in mediating PPD (Beeching *et al.*, 1997).

ROS are derived from molecular oxygen via a series of reduction reactions that alter its electron structure (Reilly *et al.*, 2004). Singlet oxygen ($^1\text{O}_2$) is formed when an unpaired electron of ground-state dioxygen is promoted to an orbital of higher energy. The superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are formed by subsequent one-electron reductions of dioxygen. While neither O_2^- nor H_2O_2 are highly reactive at steady-state levels, they act as substrates for the production of the highly reactive hydroxyl radical (OH^\bullet) via the iron-catalysed Haber-Weiss reaction (Reilly *et al.*, 2004). ROS have long been regarded as unwanted by-products of both aerobic metabolism and stress metabolism since they are able to cause widespread damage *in vivo* via the oxidation of cellular components.

ROS are normal products of metabolism and may be generated at various sites within the cell, although they are most frequently produced where there is a high rate of electron flow, such as chloroplasts and mitochondria (Mittler *et al.*, 2004). In chloroplasts, the main sources of ROS are from electron leakage from Fe-S centres of photosystem I and from the Mehler reaction (Gechev *et al.*, 2006). Stress conditions increase the rate of ROS production in these systems (Biehler & Fock, 1996). For example, high light stress increases the production of ROS from chloroplasts while other stresses result in the over-reduction of the mitochondrial electron transport chain (METC) leading to faster ROS generation. Cell wall and plasma membrane oxidases, such as NADPH-dependent oxidase, are also considered an important source of ROS (Mittler *et al.*, 2004).

Throughout their evolution, plants have developed sophisticated ROS scavenging systems to restrict cellular oxidative damage (Miller *et al.*, 2008). Various enzymatic (catalase, superoxide dismutase, ascorbate peroxidase) and non-enzymatic (ascorbate, glutathione) antioxidants are able to detoxify ROS, thereby limiting cellular oxidative damage. Carotenoids and tocopherols are able to quench $^1\text{O}_2$ directly, or alternatively they may quench excited triplet state chlorophyll in photosystem II, the major source of $^1\text{O}_2$ (Telfer *et al.*, 1994).

A balance between ROS production and ROS scavenging is crucial in avoiding over-reduction of electron transport chains and in preventing cellular oxidative damage. Furthermore, ROS have recently been implicated in functioning as plant signalling molecules, particularly in mediating stress responses (Dat *et al.*, 2000). In *Arabidopsis*, 1% of the transcriptome is regulated by H_2O_2 (Desikan *et al.*, 2001). ROS signal duration, intensity and production site are crucial

determinants in how the signal is perceived, relayed and translated into a response (Gechev *et al.*, 2006) and therefore the interplay between ROS production and scavenging is essential to ensure precise signal transduction.

1.5.3 ROS – mediators of PPD

PPD in cassava is intimately linked to the ROS production-scavenging system. Of the genes up-regulated during PPD in cassava, microarray analysis revealed eighteen percent are involved with ROS turnover (Reilly *et al.*, 2007). Additionally, above a threshold concentration a negative correlation exists between the rate of PPD and the content of antioxidant carotenoids (Sánchez *et al.*, 2006).

The initial response to wounding in cassava is the generation of large amounts of O_2^- and H_2O_2 , chiefly via NADPH-dependent oxidases (Reilly *et al.*, 2004). This can begin within fifteen minutes of harvesting, although peak levels of O_2^- and H_2O_2 occur after 4 hours and 24 hours respectively (Reilly *et al.*, 2004). The accumulation of ROS in response to a stress is a common plant wound response and is known as an 'oxidative burst' (Apostol *et al.*, 1989). While cassava storage roots are able to respond to a wounding event by exhibiting an oxidative burst, thereby switching from a state of homeostasis to one of stress, it has been theorised that they are unable to sufficiently scavenge the ROS. This prevents the switch back to homeostasis occurring and allows the oxidative burst to spread throughout the storage root from the wound site. Ultimately this leaves the storage roots in a continuous state of stress signalling, resulting in persistent ROS production. The ROS produced during the wound-induced oxidative burst may directly cause cellular oxidative damage, for example through lipid peroxidation or DNA oxidation. Alternatively, the ROS may act as signal molecules as previously described, leading to detrimental changes in ROS scavenging or wound-healing efficiency.

The role of ROS in cassava PPD is further complicated by the cyanogenic nature of the storage roots. It has been suggested that the formation of cyanogenic compounds on wounding is responsible for the oxidative burst; the cyanogenic compounds released could inhibit complex IV of the METC and thereby increase production of ROS in mechanically wounded cells (Indo *et al.*, 2007). However, this theory still relies upon the destructive nature of ROS to explain how the biochemical and molecular symptoms of PPD are able to spread throughout the root from the wound site. It should also be remembered that a

wounding event generates an oxidative burst in nearly all laboratory-tested plant species, the vast majority of which produce only very low quantities of cyanogenic compounds. It is more probable that, as well as acting as an amplifier rather than a generator of the oxidative burst, cyanogenic compounds generated at the wound site inhibit elements of the plant's antioxidant response; cyanide inhibition has been demonstrated for catalase (Ogura & Yamazaki, 1983) and superoxide dismutase (Ozaki *et al.*, 1988). This would help explain why cassava storage roots, given their cyanogenic potential, are particularly inefficient at scavenging ROS after a harvesting event.

1.5.4 ROS – mediators of programmed cell death (PCD)?

Programmed cell death (PCD) is defined as a “genetically controlled cellular suicide” (Dickman *et al.*, 2001) and is a key process in the development and maintenance of plants. It is involved in, among other things, the removal of the temporary aleurone cells, leaf senescence, the deletion of cells during plant body sculpting and the plant hypersensitive response (HR) (Dickman & Reed, 2004). PCD is much less well understood in plant systems than in animals although there are known similarities to animal PCD; the cellular morphological characteristics, changes in gene expression, the activation of proteases and DNA fragmentation or ‘laddering’.

The initial step in the majority of PCD events is the release of cytochrome c from mitochondria, which may be regulated by BCL2 protein family members. The release of cytochrome c is followed by the formation of the apoptosome and subsequently a proteolytic cascade involving cysteine proteases (caspases). The activation of ‘executioner’ caspases leads to the cleavage of critical proteins and cell death (Hoeberichts & Woltering, 2003; Dickman & Reed, 2004; Reape *et al.*, 2008).

In plants there are few genes with homologous sequences to identified animal PCD genes – one known example is the *Arabidopsis* homologue of human *Bax inhibitor-1* (*AtBI1*) (Sanchez *et al.*, 2000) whose over-expression led to reduced levels of PCD after chemical treatment (Watanabe & Lam, 2008). The introduction of animal anti-apoptotic genes into plant systems has repeatedly resulted in a reduction of cell death and an increased resistance to stress (Mitsuhara *et al.*, 1999; Dickman *et al.*, 2001; Kawai-Yamada *et al.*, 2001; Xu *et al.*, 2004). This suggests the molecular mechanisms for plant PCD and animal

PCD have been evolutionarily conserved. Plant functional homologues of animal caspases do exist and are termed metacaspases. The over-expression of *Arabidopsis Metacaspase-8 (AtMC8)* yielded lines hypersensitive to PCD induction, while knockout lines showed reduced levels of PCD (He *et al.*, 2008).

In plant systems, one of the most characterised stress responses is the HR, describing the localised activation of PCD pathways around the site of pathogen entry into plant tissues. The products of pathogen-derived avirulence (*avr*) genes are recognised by plants possessing the corresponding resistance (*R*) gene leading to the induction of the HR and the physical restriction of the invading pathogen (Morel & Dangl, 1997). One of the earliest events of the HR after pathogen recognition is the rapid production of ROS (oxidative burst). These ROS may be capable of causing lipid peroxidation leading to membrane damage and cell death, or alternatively the ROS may act as signal molecules capable of activating PCD pathways. It is also possible that, since the release of cytochrome c from the mitochondrion can lead to the inhibition of electron flow between complex III and complex IV of the METC, the subsequent ROS production acts as a PCD amplification mechanism (Lam *et al.*, 2001). Furthermore, the release of cytochrome c from mitochondria has been shown to be ROS-dependent in a plant cell culture system using *Nicotiana tabacum* Bright-Yellow 2 cells (Vacca *et al.*, 2006).

Currently, it is not known whether cassava storage roots undergo PCD, although some form of cell death does occur. Given that ROS play key roles in the HR and that cassava experiences a persistent oxidative burst on harvesting, it is not unreasonable to suggest that PPD and PCD may be intimately linked. Microarray data comparing cassava transcripts pre- and post-harvest identified four genes potentially involved in regulating PCD pathways (Reilly *et al.*, 2007). Two novel genes that may act as PCD enhancers, a cysteine protease and a class IV chitinase, were significantly up-regulated after harvesting while two potential suppressors of PCD, a cystatin-like protein and a translationally controlled tumour protein, were significantly down-regulated after harvesting. Additionally, PCD-related sequences with high homology to pea *Cytochrome P450 Monooxygenase*, tobacco *Peroxidase* and citrus *Defender Against Cell Death 1 (Dad1)* were identified as up-regulated post-harvest using cDNA-AFLP analysis (Huang *et al.*, 2001).

1.6 Post-harvest deterioration of tropical tuber crops

PPD in cassava is a purely physiological process. A persistent oxidative burst brings about widespread cellular oxidative damage and may also act as a signal to activate other pathways, such as PCD. Other tuberous crops are more efficient at forming a wound periderm and healing than cassava. This removes the entry point for oxygen into the tuberous tissue and helps prevent further oxidative damage and allows recovery from the wounding event. Consequently, the primary mechanism of post-harvest deterioration is often different in these crops and the shelf-life considerably longer. By assessing the properties of crop tubers with a shelf-life longer than cassava, it may be possible to identify factors that are important in causing PPD in cassava storage roots.

1.6.1 Sweet potato (*Ipomoea batatas*)

The average storage time of sweet potatoes is approximately 2-3 weeks, but may be several months if the storage roots are handled and stored correctly. As in cassava, deterioration of the sweet potato (*Ipomoea batatas*) is heavily influenced by mechanical damage sustained during harvesting, transport or storage. Approximately 25% of storage roots are damaged during harvesting itself, although this will vary with variety and the technique used (Ray & Ravi, 2005). In sweet potatoes, mechanical damage induces an increase in the respiration rate, leading to dehydration and weight reduction (Picha, 1986) and resulting in shrivelling on the outer surface of the tuber. Ethylene (Inaba *et al.*, 1989) and high oxygen levels (Chang & Kays, 1981) enhance the respiration rate, the latter suggesting a potential for the involvement of ROS signalling during sweet potato deterioration. The presence of open wounds appears conducive to high levels of respiration since once wounds are healed respiration falls steadily (Picha, 1986). In contrast to cassava, discolouration is only linked with microbial infection and not with any plant phenolic reactions in sweet potato.

Importantly, wounds in sweet potato appear to be healed more readily and with more success than in cassava. This may be due to a single ROS burst, rather than a continuous oxidative signal as is seen in cassava, which allows the mechanisms involved in wound healing to operate efficiently. Alternatively, wound healing may be the trigger for the down-regulation of the oxidative response and a return towards homeostatic conditions without major tissue damage. Lower generation of cyanogens after harvest may also aid wound-healing in sweet

potatoes. The precise ROS response in sweet potatoes is not known but in either case, the extensive oxidative damage seen in cassava storage roots post-harvest is frequently not seen in sweet potatoes giving them a longer shelf-life. Therefore, other mechanisms of deterioration are considered more serious than wounding.

Since sweet potatoes act as propagules, sprouting of sweet potatoes is a substantial source of physiological post-harvest loss. Sprouting of tubers can occur before harvest when soil moisture is high and also after harvest when temperature and relative humidity (RH) are high (Ray & Ravi, 2005). During sprouting, organoleptic qualities of the roots decline and the sugar content of the tuber may fall by up to 50%, further resulting in a loss of eating quality (Ravi & Aked, 1996). For this reason stored tubers are routinely checked for newly formed sprouts, which are broken off as they emerge.

Mechanical damage supplies points of entry for pathogenic bacteria and fungi and the high RH often achieved during storage promotes microbial decay. Soft rot is a global constraint to sweet potato storage caused by fungi of the species *Rhizopus stolonifer*, *R. oryzae* and *R. nigricans* (Ray & Ravi, 2005). The disease usually brings about total rotting of tubers in 3-4 days and can spread between individual tubers in storage. Bruising is the injury type most likely to incite soft rot infection (Holmes & Stange, 2002) and so care during handling is essential. Other important forms of sweet potato storage rot include sclerotium rot (*Sclerotium rolfsii*), bacterial rot (*Erwinia chrysanthemi*), spongy rot (*Cochliobolus lunatus*) and rhizoctonia rot (*Rhizoctonia solani*) (Ray & Ravi, 2005). Other bacterial and fungal species do cause disease prior to harvesting that lead to post-harvest losses, although they will not be discussed here.

In several tropical regions, weevils are considered the biggest constraint to sweet potatoes post-harvest. Losses typically range between 30-75%, with cured roots having no apparent resistance to attack. Sweetpotato weevils (*Cylas formicarius* and to a lesser extent *C. puncticollis* Boheman and *C. brunneus*) prefer storage roots to other tissues and use them for oviposition and as a food source (Ray & Ravi, 2005). The tubers may respond by producing defensive phytoalexins such as ipomeamarone (Imaseki *et al.*, 1964), rendering them odorous and unpalatable. There are over 80 arthropod species in total that infest sweet potatoes in storage, although the sweet potato weevil is the principal pest. Rodents and nematodes may also contribute towards post-harvest losses although their impact is negligible in comparison (Ray & Ravi, 2005).

1.6.2 Yam (*Dioscorea* spp.)

Deterioration in yams is highly analogous to that in sweet potato. A major source of post-harvest loss is caused by an increase in respiration leading to a reduction in weight and a loss of marketability (Passam *et al.*, 1977; Ravindran & Wanasundera, 1992). The sealing of wounds via curing suppresses the respiration rate and water loss, allowing storage for several months. The curing of yams is dependent on temperature, RH (Passam *et al.*, 1976) and possibly light irradiation. Tubers cured in red light exhibited 80% rotting after 24 weeks of storage whereas tubers cured under green, blue or yellow light showed none (Ravi *et al.*, 1996). Physical injury through improper handling causes further increases in respiration and may provide points of entry for pathogenic microbes. Rotting via microbes is generally rapid and may spread to healthy tubers if storage is dense.

As in sweet potatoes, sprouting is a major source of storage losses in yam. Yams enter a state of dormancy after harvesting when the metabolic activity of the tubers is reduced to a minimum (Passam & Noon, 1977). It has been demonstrated that temperatures around 15 °C can delay sprouting regardless of RH, although such temperatures are not ideal for curing and are hard to achieve in practice in tropical regions. Endogenous phenolic growth inhibitors termed batatasins are responsible for inducing and maintaining dormancy and only when batatasin levels fall do yams begin to sprout. Once sprouting has been initiated, the tuber begins to senesce rapidly with available nutrients used for growth. As a result, sprouting ultimately leads to tuber deterioration (Passam & Noon, 1977).

Yams are susceptible after harvest to a similar array of biotic agents as sweet potatoes, including bacteria, fungi (e.g. *Aspergillus niger*), viruses (e.g. yam mosaic potyvirus), nematodes (*Scutellonema bradys*) and arthropods (*Palaeopus dioscoreae*) (Jansson, 1992; Kenyon *et al.*, 2000; Kwoseh *et al.*, 2000; Okigbo, 2005).

1.6.3 Cocoyams (*Xanthosoma sagittifolium* and *Colocasia esculenta*)

The cocoyams are edible aroids and comprise new cocoyam (tannia, *Xanthosoma sagittifolium*) and old cocoyam (taro, *Colocasia esculenta*). In cocoyams, the initial mechanism of deterioration is weight loss through respiration. As in sweet potato and yam, high RH (85%) during storage acts to reduce respiration and weight loss (possibly through curing) although in contrast, an intermediate (15 °C) rather than a high temperature was required. Respiration is accompanied by the conversion

of starch to sugars further detracting from the quality of the crop. High RH also increases the rate of sprouting and microbial infection of stored taro and tannia. The respiration rate of tannia after harvesting is consistently less than that of taro possibly explaining the longer shelf-life of tannia (Agbor-Egbe & Rickard, 1991). The removal of cornelets, or secondary cormels, in some taro and tannia varieties on harvesting leads to the production of more wound sites and therefore higher respiration rates and more points of entry for microbial pathogens (Agbor-Egbe & Rickard, 1991). Hence varieties with cornelets tend to deteriorate faster.

Sprouting is a source of loss in cocoyams after harvesting. Dormancy is not well studied in cocoyams and no experimental evidence for its existence has been provided. It is thought that if cocoyams do enter a dormant phase, it lasts very briefly (Ravi & Aked, 1996) and so sprouting may occur early during storage.

Microbial invasion and subsequent rotting account for a considerable proportion of post-harvest losses of cocoyams, as they do for the other crops mentioned here. Cocoyams are susceptible to a similar array of fungal rots as sweet potato (Gollifer & Booth, 1973). The invasion of the tubers by microbes is facilitated by mechanical damage incurred during harvesting, transportation or storage.

1.6.4 Implications for cassava

While cassava storage roots have a beneficial role in supporting recovery of the plant after periods of drought and defoliation, they cannot, unlike all other tuberous crop species mentioned here, act as propagules and serve no function once detached. Therefore, there is no selective advantage to the plant to repair wounds and sustain storage roots once detached from the mother plant. Ultimately this renders cassava storage roots particularly vulnerable to rapid physiological deterioration post-harvest while other tuberous crop species deteriorate more slowly. Wound healing has been observed in storage roots still attached to the mother plant in response to fungal infection, with an absence of wound repair in detached storage roots (Mwenje *et al.*, 1998). This may indicate that the resources necessary for efficient wound repair cannot be provided solely by the storage roots.

Furthermore, the storage roots of sweet potato, yam, cocoyam and also potato all contain storage proteins. A secondary role in wound defence has been inferred for these storage proteins since they may be induced by wounding and

have antioxidant enzymatic activity (these proteins are described in more detail in Section 4.3.1) (Andrews *et al.*, 1988; Hou & Lin, 1997; Hou *et al.*, 1999; Hou & Lin, 2002; Shewry, 2003). To date, the presence of a specific storage root protein has not been established in cassava storage roots. It may be that the absence of a specific wound-inducible antioxidant protein renders the storage roots of cassava more susceptible to the downstream effects of wounding than the other tuberous crops.

1.7 Methods for delaying PPD

1.7.1 Pre-harvest methods

Harvesting causes severe mechanical damage to cassava storage roots, some of which could be avoided. Selecting varieties with compact, short roots rather than long dispersed roots can reduce mechanical damage triggered by both digging and removal of roots from the ground, thereby delaying wound-induced deterioration (Booth, 1976; Rickard & Coursey, 1981).

Pruning of the foliage 2-3 weeks prior to harvest delays deterioration; for example in the MCOL 22 variety, storage roots stored for 20 days exhibited 96% deterioration without pruning but only 4% deterioration when pruned three weeks before harvest (Rickard & Coursey, 1981). The exact mechanism at work here is not known although pruning results in a decrease in the respiration rate, weight loss and PAL activity of stored roots (Data *et al.*, 1984; Tanaka *et al.*, 1984). Anecdotes suggest pruning negatively affects the organoleptic quality of cassava storage roots and reduces the starch content and so is often avoided in the field (Reilly *et al.*, 2004). Other reports indicate that if the pruning-harvest interval is 1-3 weeks there is no substantial difference in the texture, flavour or pasting properties of the starch, although there is a reduction in the total starch content (van Oirschot *et al.*, 2000).

1.7.2 Simple/traditional methods

The simplest method for delaying post-harvest deterioration is to avoid harvesting altogether. By leaving the roots in the ground until needed, the mechanical damage caused by harvesting that instigates the oxidative burst does not occur (Rickard & Coursey, 1981). While this is effective for small-scale local production it is ineffective when the cassava is produced for the market place. Additionally, it

leaves the cassava plants susceptible to a wide array of pests and diseases that can detract from the storage root quality. Furthermore, if left in the ground for too long the yield falls and storage roots become woody with an impaired flavour (Westby, 2002). In-ground storage also uses land that could be employed for growing additional crops – if half the 18.5 million hectares of cassava grown in 2004 were left in the ground for two months, over 1.5 million hectares of land would have been occupied needlessly (Montaldo, 1973; FAOSTAT, 2009).

Other traditional methods are often based on oxygen exclusion techniques. Wrapping roots in plastic, dipping in paraffin wax or storage in controlled environments can lead to a 4 week shelf-life but are high-cost methods and therefore suitable only for storage root export to developed countries since cassava is a low-cost commodity in the countries of origin (Rickard & Coursey, 1981; Reilly *et al.*, 2004).

Under conditions of high humidity and temperature, cassava storage roots can form a wound periderm and heal by a process known as curing that also occurs in other root crop species (Reilly *et al.*, 2004). The desiccation of parenchyma cell layers is followed by deposition of suberin/lignin on cell walls around the wound. This leads to meristem formation and the subsequent development of the wound periderm (Booth, 1976). Clamps take advantage of this aspect of cassava physiology and permit storage for up to two months – storage roots are piled onto a bed of straw and then covered with more straw and soil (Rickard & Coursey, 1981). The temperature and RH inside the clamp is crucial for curing and this is controlled by ventilation of the clamp. While effective at delaying deterioration this technique is variable and unreliable, possibly due to the inefficient curing seen in cassava storage roots (Booth, 1976).

1.7.3 Processing

Approximately half of all cassava intended for human consumption undergoes a form of processing (Salunkhe & Desai, 1984). The roots can be dried producing chips, flours and starch, or alternatively soaked or fermented. As well as having a reduced cyanogenic content, these products are less susceptible to deterioration than unprocessed roots but are sensitive to fungal contamination (Westby, 2002). That said, a well-prepared gari can stand for several months without decay or fungal attack (Salunkhe & Desai, 1984).

Large scale processing of cassava storage roots for human consumption or industrial products is more complex. Long distances between producers and processors permit the initiation of PPD before the storage roots have been converted to a more stable form (Reilly *et al.*, 2004). Furthermore, an increase in labour input drives price increases for cassava products.

1.7.4 Breeding

Cassava is consecutively monoecious and demonstrates limited flowering (Jennings & Iglesias, 2002). It is also highly heterozygous, which makes recovering improved lines or farmers' preferred varieties through back-crossing virtually impossible. Additionally, the multiplication rate of cassava via vegetative propagation is slow and the seed set is on average only 0.6 per pollination (Ceballos *et al.*, 2004). These factors make controlled breeding programmes laborious and complex although there have been some notable successes. Work by the International Institute of Tropical Agriculture (IITA) led to the development of cassava lines with enhanced resistance to cassava mosaic disease (CMD) that have subsequently been distributed throughout many cassava-producing countries (Legg & Fauquet, 2004). Cassava has also been crossed with its wild relative, *Manihot glaziovii*, to introduce genes for apomixis into the crop variety, allowing the maintenance of superior varieties (Nassar & Collevatti, 2005).

Unfortunately, advantageous traits are often recessive and tightly genetically linked to disadvantageous ones making trait separation awkward. There is a strong genetic link between PPD and the advantageous trait of dry matter content (Jennings & Iglesias, 2002) making delaying PPD through breeding challenging. As a result, few breeding programmes have been initiated with the aim of delaying PPD, although one has indicated it is possible to break the association between high dry matter content and PPD (Jennings & Iglesias, 2002). The screening of numerous cassava varieties and improved lines for nutritional content and agronomic traits has been carried out and provides a solid platform to move forward with cassava breeding (Chávez *et al.*, 2005).

Another strategy that could facilitate or accelerate a breeding programme for delaying deterioration is marker-assisted selection (MAS). While attempts have been made to isolate quantitative trait loci for PPD, few markers linked to major loci have been identified (Cortés *et al.*, 2002). Varieties with low levels of PAL expression or high levels of antioxidant gene expression post-harvest could

be identified and used as parents in crosses. However, the PPD phenotype exhibits a high 'genetic X environment' interaction that makes scoring for minor differences difficult (Rodríguez, 2001).

1.7.5 Biotechnology

Genetic engineering provides the most powerful tool for modulating post-harvest deterioration in cassava roots. The use of tissue- or developmentally-specific promoters to drive the expression of target genes can mediate significant physiological changes *in planta*. These genetic alterations are usually initiated via *Agrobacterium tumefaciens*-mediated transformation events in cassava (Li *et al.*, 1996; Schreuder *et al.*, 2001). Since the modulation of expression of a single gene is achievable, tightly linked traits such as PPD and dry matter content may be modified independently. Other benefits are that transgenic lines with improved qualities can be stably maintained through vegetative propagation since gene segregation through out-crossing is restricted (Fregene & Puonti-Kaerlas, 2002; Ihemere *et al.*, 2006) and that transgenes can be directly introduced to farmer-preferred varieties maintaining desirable eating qualities (Masona *et al.*, 2001). Genetic engineering of crops in Africa and other developing countries for the benefit of the farmer and consumer is generally seen as a potential positive influence on food availability as opposed to the attitude prevalent in wealthier nations, especially in Europe.

The genetic modification of cassava for improved agronomic and other characters is in its infancy, due largely to the cost involved, the necessary infrastructure and expertise required and the lack of importance of this crop to developed nations. Three notable exceptions are: the modification of cassava for enhanced starch production by enhancing storage root ADP-glucose pyrophosphorylase activity, which led to a higher yield through an increase in root size and number (Ihemere *et al.*, 2006); the over-expression of hydroxynitrile lyase in cassava plants, which led to a reduction in the content of the harmful acetone cyanohydrin (Siritunga *et al.*, 2004); and the over-expression of a cassava mosaic virus gene encoding the replication-associated protein (AC1) in cassava, which led to increased silencing of the viral AC1 gene and conferred resistance to cassava mosaic geminiviruses (Chellappan *et al.*, 2004). There is currently a wide array of active cassava genetic engineering projects with the aim of generating material with improved nutritional value for agricultural use in cassava-producing countries.

The BioCassava Plus project (www.biocassavaplus.org) is seeking to enhance nutrient content, increase virus resistance, reduce cyanogenesis and delay post-harvest deterioration in cassava storage roots through genetic engineering. For the latter, the over-expression of cassava antioxidant genes under the control of a root-specific promoter may help to reduce the negative impact of ROS after harvesting. Other areas of focus for increasing shelf-life through genetic engineering include the modification of programmed cell death pathways in cassava and modulation of the phenolic content of storage roots. A key factor in the success of extending the shelf-life of cassava is the transfer of genetic engineering techniques and technologies to the cassava-producing countries. This would lessen the distance between technology and consumer, ensuring that genuine rather than apparent problems are being addressed (Masona *et al.*, 2001).

1.8 Research strategy

PPD in cassava is regarded as an ROS-mediated process since the ability of storage roots to efficiently scavenge ROS after harvesting is compromised when compared to wound responses in other plant species. Therefore, this research aimed to increase the capacity of storage roots to scavenge ROS through the modulation of individual components of the ROS-scavenging machinery using a biotechnological approach. Three genes encoding antioxidant enzymes were selected for modulation of expression: *APX*, *CAT* and *SOD*. The enzymes these genes encode are major participants in ROS removal in plant systems and furthermore versions of these genes have been isolated and sequenced in cassava. The *StPAT* promoter, isolated from potato tubers, was used to drive the expression of the target genes in a root-specific manner.

1. The initial phase of this investigation was concerned with the design and production of expression plasmids. The aim was to generate plasmids that would confer high and stable target gene expression in transgenic plants as well as a reporter expression plasmid that would facilitate the characterisation of the *StPAT* promoter.
2. The second goal of this research was to determine the expression pattern of the *StPAT* promoter in transgenic *Arabidopsis* lines carrying the reporter

expression plasmid. This would provide a model system to confirm the root-specificity of expression conferred by the *StPAT* promoter. Additionally, if the promoter was shown to drive target gene expression in a root-specific manner, then *Arabidopsis* could subsequently be used as a model to test the target gene expression plasmids.

3. To establish the effect of root-specific target gene over-expression, expression plasmids were then used to transform the model plant *Arabidopsis thaliana*. This would indicate the level of target gene expression that might be expected in transgenic cassava storage root tissue. Transgenic *Arabidopsis* plants were then analysed to determine if an increase in antioxidant enzyme activity could increase their resistance to oxidative stress.
4. The final aim was to generate cassava plants transformed with the expression plasmids and assess the effect of target gene expression on PPD.

2 MATERIALS AND METHODS

All chemicals and equipment were obtained from Sigma Aldrich unless otherwise stated.

2.1 MATERIALS

2.1.1 Bacterial strains

The *Escherichia coli* strains used for cloning purposes were One Shot® TOP10 (Invitrogen), DB3.1™ (Invitrogen) and home-made DH5α (Hanahan *et al.*, 1991) chemically competent cells.

Arabidopsis was transformed using homemade *Agrobacterium tumefaciens* GV3101 electrocompetent cells harbouring the chromosomal C58 background (rifampicin resistance) and the cured pGV3101 Ti plasmid (gentamycin resistance) (Holsters *et al.*, 1980; Koncz & Schell, 1986). Tobacco and cassava were transformed using homemade *A. tumefaciens* LBA4404 electrocompetent cells. This strain harboured the chromosomal TiAch5 background (rifampicin resistance) and the pAL4404 Ti plasmid (streptomycin resistance) (Hoekema *et al.*, 1983).

2.1.2 Plasmids

The pCR®2.1-TOPO® plasmid (Invitrogen) was used for subcloning of target genes and the *StPAT* promoter. In addition to TA cloning capability, this plasmid possessed both ampicillin and kanamycin resistance genes as well as the *LacZα* fragment for blue/white selection.

Target genes with *attB* Gateway® tags were recombined into pDONR™/Zeo (Invitrogen). This plasmid carried the Zeocin™ resistance gene for bacterial selection. It also contained the *ccdB* gene, which interfered with *E. coli* DNA gyrase and so inhibited growth of colonies (Bernard & Couturier, 1992). Successful recombination of target genes removed the *ccdB* gene allowing high-efficiency recovery of desired clones. pDONR™/Zeo plasmids with target genes successfully recombined were termed pENTR™ plasmids.

pCAMBIA 1305.1 was procured from the Centre for the Application of Molecular Biology to International Agriculture (CAMBIA, <http://www.cambia.org>, GenBank accession: AF354045) and was used as a template to create all the

variations of expression/destination plasmid. This binary vector featured the hygromycin resistance gene (*hptII*) for plant selection and the *GusPlus* (*GusP*) reporter gene (Broothaerts *et al.*, 2005) under the control of the constitutive CaMV35S promoter within the T-DNA region. It also harboured the kanamycin resistance gene in the vector backbone for bacterial selection. pENTR™ plasmids were recombined with a modified version of pCAMBIA 1305.1 (designated pCAM PAT GW) that included the *StPAT* promoter in front of the Gateway® cassette, in place of *CaMV35S::GusP*. A reporter construct (designated pCAM PAT *GusP*) was also created with the *StPAT* promoter driving expression of *GusP*. The manipulation of pCAMBIA 1305.1 is described in Chapter 3.

2.1.3 Plant material

Wild-type *Arabidopsis thaliana* Col-0 seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The ethylene-insensitive mutant *etr1-1* was kindly donated by Professor Mike Hall (Institute of Biological, Environmental and Rural Sciences, Aberystwyth University) and the jasmonic acid-insensitive mutant *jin1-1* was received from Professor Barbara Kunkel (Washington University, St. Louis). Wild-type tobacco SR1 seeds were provided by Julia Watling (University of Bath). For cassava work, TMS 60444 plants grown in the University glasshouse were used unless otherwise stated.

2.1.4 Software

Virtual cloning was carried out using Vector NTI 10 (Invitrogen). The same software was used for assembling contigs following DNA sequencing and for all sequence alignments. Primers for sequencing, PCR and sqPCR were designed using the NetPrimer online tool (www.premierbiosoft.com). *cis* regulatory motifs were identified by submitting promoter sequences to the PLACE (<http://www.dna.affrc.go.jp/PLACE/>) and PlantCARE (<http://bioinformatics.psb.ugent.be>) online databases (Higo *et al.*, 1999; Lescot *et al.*, 2002). The identification of conserved *cis* regulatory motifs in co-regulated promoters was performed using the Weeder web tool (<http://159.149.109.9/modtools/>) (Pavesi *et al.*, 2006). SignalP 3.0 (<http://www.cbs.dtu.dk>) and the Signal Peptide database (<http://proline.bic.nus.edu.sg/spdb/>) were used to locate and characterise signal

peptide sequences in the GusP protein (Bendtsen *et al.*, 2004; Choo *et al.*, 2005). Cary WinUV software (Varian) was employed to record and analyse the data from the ascorbate peroxidase and catalase assays. The leaf area, root length and root growth angle of *Arabidopsis* seedlings was determined using ImageJ 1.41 (<http://rsb.info.nih.gov/ij/>). Statistical analyses were carried out using Minitab 15. *Arabidopsis* microarray data was provided by Genevestigator V3 (Hruz *et al.*, 2008) with data from 4,069 ATH1 22K arrays assessed in each instance.

2.2 METHODS – Growth and transformation of bacteria and plants

2.2.1 Antibiotics for bacterial and plant selection

Information concerning antibiotics used for the selection of desired bacterial clones and plant lines are given below (Table 2.1). Hygromycin B and Augmentin™ were sourced from Melford and Zeocin™ was included with the pDONR™/Zeo plasmid (Invitrogen).

Table 2.1: Antibiotic preparation.

Antibiotic	Prepared In	Sterilisation	Working Concentration
Augmentin™	SDW	filter sterilisation	400 µg/ml
Gentamycin	SDW	filter sterilisation	25 µg/ml
Hygromycin B	SDW	filter sterilisation	50 µg/ml
Kanamycin	SDW	filter sterilisation	50 µg/ml
Rifampicin	100% methanol	None	100 µg/ml (GV3101) 50 µg/ml (LBA4404)
Streptomycin	SDW	filter sterilisation	100 µg/ml
Zeocin™	SDW	filter sterilisation	50 µg/ml

2.2.2 Preparation of competent bacteria

10 ml of liquid LB media was inoculated with a single colony (of *E. coli* DH5α, *A. tumefaciens* GV3101 or *A. tumefaciens* LBA4404) and incubated at 37 °C for 16 hrs with shaking. 250 µl of culture was used to inoculate 50 ml liquid LB media and the second culture was shaken until the A₆₀₀ reached 0.48. After a 30 min incubation on ice to stop cell division, the culture was centrifuged at 1,388 x g for

10 minutes at 4 °C in a Universal 32R centrifuge (Hettich) and the supernatant discarded. Bacterial pellets were gently resuspended in ice-cold 0.1 M calcium chloride, incubated on ice for 30 mins, centrifuged as before and the supernatant discarded. This process was repeated twice more, resuspending first in 8 ml ice-cold 0.1 M calcium chloride and then 2 ml ice-cold 100% glycerol. 50 µl of cells were aliquoted into chilled tubes, snap-frozen in liquid nitrogen and stored at –80 °C.

2.2.3 Growth of *E. coli* and *A. tumefaciens*

E. coli liquid cultures were grown in 5 ml liquid LB media inoculated with single colonies and containing the appropriate antibiotic. Liquid cultures were grown for 16 hrs at 37 °C, with shaking at 200 rpm. *E. coli* colonies were cultivated on LB agar plates containing the appropriate antibiotic. Plates were incubated for 16 hrs at 37 °C.

A. tumefaciens liquid cultures and colonies were grown in the same way, with the incubation temperature reduced to 28 °C and the incubation time increase to 40 hrs.

2.2.4 Transformation of chemically competent cells (*E. coli*)

Chemical transformation was performed using the heat-shock method. DNA was combined with a 50 µl aliquot of cells and incubated on ice for 30 mins. A heat-shock treatment at 42 °C for 30 secs was administered and the cells returned to ice for 2 mins. 500 µl sterile LB media was added to the samples, which were then incubated at 37 °C with shaking for 1 hr. 100 µl of cells were spread onto LB agar plates (+ antibiotics) and incubated for 16 hrs at 37 °C.

2.2.5 Transformation of electrocompetent cells (*A. tumefaciens*)

A. tumefaciens cells were transformed through electroporation. DNA was mixed with a 50 µl aliquot of cells and immediately transferred to an ice-cold electroporation cuvette (Bio-Rad, 2 mm gap). Electroporation was carried out using a MicroPulser (Bio-Rad) on the Ec2 setting (2.5 kV, 1 pulse). Directly after pulsing, 750 µl sterile LB media was added to the cells, which were then incubated

at 28 °C with shaking for 2 hrs. 100 µl of cells were spread onto LB agar plates (+ antibiotics) and incubated for 40 hrs at 28 °C.

2.2.6 Growth of *Arabidopsis* and cross-pollinations

All *Arabidopsis* seed was surface sterilised prior to sowing by washing once with 70% ethanol, once with 50% bleach, 0.05% Tween20 and six times with sterile SDW. Seed samples were stratified at 4 °C in sterile 0.15% agar for 3 days.

Soil-grown *Arabidopsis* plants were cultivated in Levington's F2 compost pre-treated with 0.02% Intercept 70WG (Scotts). Plants were grown in a controlled temperature room (Sanyo) at 22 °C and 60% relative humidity with a day length of 16 hrs.

Plate-based *Arabidopsis* growth occurred on sterile MS media (4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 1% sucrose and 0.3% Gelrite™ (Melford), pH adjusted to 5.8). Plates were sealed with one layer of micropore tape (3M). Plates were incubated in a controlled temperature room (Sanyo) at 22 °C and 60% relative humidity with a day length of 16 hrs. To harvest roots, 100 mm square plates were incubated standing 5° from vertical to encourage root growth along the media surface.

Cross-pollinations were carried out by removing the anthers of closed flower buds of the seed parent 1 day prior to anthesis. Two days later, the anthers of open flowers from the pollen parent were gently touched onto the stigma of the seed parent. Cross-pollinated flowers were clearly labelled and allowed to set seed.

2.2.7 Transformation of *Arabidopsis* and selection of single-insertion homozygous lines

Arabidopsis thaliana Col-0 plants were transformed according to the *Agrobacterium*-mediated floral dipping method (Clough & Bent, 1998). Each transformation was performed on approximately 20 wild-type Col-0 plants (T₀). A 50 ml liquid LB starter culture (+ antibiotics) was inoculated with a single colony of *A. tumefaciens* GV3101 harbouring a destination/expression plasmid. After a 16 hr shaking incubation at 28 °C, 10 ml of starter culture was used to inoculate 500 ml liquid LB media (+ antibiotics). This second culture was incubated for 16 hrs at

28 °C with shaking. Cells were harvested by centrifuging at 4,615 x g for 15 mins in an Avanti J-26 XP centrifuge with JA-25.50 rotor (Beckman Coulter). Cell pellets were resuspended with 500 ml fresh 5% sucrose solution and 180 µl Silwett L-77 surfactant (Lehle Seeds). Flowers were dipped in the cell suspension for approximately 10 secs. T₀ plants were placed in a dark, humid environment for 16 hrs, then returned to the controlled temperature room and allowed to set seed (T₁). T₁ seeds were selected on MS media containing 50 µg/ml hygromycin B to identify positive transformants (Harrison *et al.*, 2006). Positive T₁ plants were grown on and the resulting seed (T₂) collected.

Single-insertion lines were identified by plating 100 T₂ seeds on MS media with 50 µg/ml hygromycin B. Lines showing 75% resistance to hygromycin B were assumed to be single-insertion. A small number of these plants were grown on and allowed to set seed (T₃). Approximately 40 T₃ seeds from each T₂ line were plated on MS media with 50 µg/ml hygromycin B and those showing 100% resistance to the selection agent were assumed to be homozygous. Single-insertion, homozygous T₃ seed was used for all downstream experiments.

2.2.8 Growth of tobacco

Soil-grown tobacco (*Nicotiana tabacum* SR1) plants were cultivated in Levington's C2 compost. Plants were grown at approximately 26 °C in a glasshouse with supplemented lighting.

In vitro-grown tobacco was cultivated in sterile MSO media (4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 3% sucrose, 0.3% Gelrite™ (Melford), pH adjusted to 5.8) in 330 ml clear plastic pots (Greiner). Pots were sealed with one layer of micropore tape (3M) and plants were grown in a controlled temperature room (Sanyo) at 28 °C with 45% relative humidity and a 16 hr day length.

2.2.9 Transformation of tobacco

5 ml of liquid LB media was inoculated with a single colony of *A. tumefaciens* LBA4404 harbouring a destination/expression plasmid. The culture was incubated at 28 °C with shaking for 24 hrs. Newly expanded tobacco leaves were excised from the mother plant and cut into 1 cm² discs, discarding the mid vein. The discs were surface sterilised by washing once in 10% bleach and four times in SDW.

Sterile leaf discs were immersed in 20 ml liquid MSO media (4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 3% sucrose, pH adjusted to 5.8) with 2 ml culture for 20 mins. The leaf discs were then plated onto sterile MSD4x2 media (4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 3% sucrose, 0.3% Gelrite™, 540 nM NAA, 4.4 µM BAP, pH adjusted to 5.8) and grown under low light intensity at 25 °C to allow shoots to form. Three days later, the discs were transferred to sterile MSD4x2 media containing 50 µg/ml hygromycin B (for selection of positive transformants) and 400 µg/ml Augmentin™ (to kill any remaining *Agrobacteria*). When shoots were large enough, they were transferred to solid MSO media (to permit rooting) containing hygromycin B and Augmentin™ and grown under high light intensity at 28 °C.

2.2.10 Growth of cassava

Cassava (*Manihot esculenta* TMS 60444) plants were grown in a 60% Levington's C2 compost, 40% perlite mix. Osmocote slow release fertiliser (Scotts) was added to the compost mix when plants had become established. Cassava was grown in a glasshouse with supplemented lighting at 30 °C during the day and 17 °C at night.

2.2.11 Transformation of cassava

The introduction of transgenes into cassava was achieved using *Agrobacterium*-mediated transformation of friable embryogenic calli (FEC) using a method similar, but containing important changes, to that of Schreuder *et al.*, 2001. All cassava transformations and analysis of potential transgenic lines was performed by Simon Bull at ETH, Zürich.

Production of friable embryogenic clusters: Young stem cuttings of cassava TMS 60444 were placed on sterile CAM (Cassava Axillary Bud Media; 4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 2 µM CuSO₄, 2% sucrose, 0.3% Gelrite™, 1 mg/L BAP) and incubated at 28 °C in the dark to induce axillary bud formation. After 2-3 days buds were removed and placed on sterile CIM (Cassava Induction Media; 4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 2 µM CuSO₄, 2% sucrose, 0.3% Gelrite™, 12 mg/L picloram) and incubated at 28 °C in the dark. Every two weeks, for a total of

approximately 6-8 weeks, the developing embryos were sub-cultured onto fresh CIM. Embryonic clusters were then placed on sterile GD media (4.4 g/L Gresshof and Doy salts with Gamborg's vitamins (Melford), 2% sucrose, 0.3% Gelrite™, 12 mg/L picloram) and incubated for approximately 3 weeks at 28 °C in the dark. At this time friable embryonic calli (FECs) should be visible and subsequently removed to fresh GD media and incubated at 28 °C in the light. Every two weeks the FECs were sub-cultured and any soft callus tissue was removed. After several cycles on GD media the FECs were suitable for *Agrobacterium*-mediated transformation.

Agrobacterium-mediated FEC transformation: A single colony of *A. tumefaciens* LBA4404 containing the desired expression construct was isolated from a YEB plate (5 g/L bacto-beef extract, 1 g/L bacto yeast extract, 5 g/L bacto peptone, 5 g/L sucrose, 15 g/L bactoagar, pH adjusted to 7.2) and used to inoculate 5 ml of sterile liquid YEB media (5 g/L bacto-beef extract, 1 g/L bacto yeast extract, 5 g/L bacto peptone, 5 g/L sucrose, pH adjusted to 7.2, 2 ml 1 M MgSO₄ added following autoclaving) containing 100 µg/ml streptomycin, 50 µg/ml kanamycin and 25 µg/ml rifampicin. The culture was incubated at 28 °C (250 rpm) for approximately 36 hrs. Cultures were used to inoculate 25 ml of sterile liquid YEB media containing antibiotics and incubated as before until the A₆₀₀ reached 0.5-1.0. Cultures were centrifuged at 1,388 x g for 10 mins in a Universal 32R centrifuge (Hettich) and resuspended in 25 ml of sterile liquid GD media (pH 5.8). Samples were centrifuged again as above and the supernatant discarded. The pellet was resuspended in liquid GD media until the A₆₀₀ reached 0.5. 200 µM of acetosyringone was added and samples gently shaken (50 rpm) for 45 mins.

The cultures were dropped onto the FECs and co-cultivated under bright light (16 hrs light / 8 hrs dark), 22 °C for 3 days. FECs were removed and washed several times with approximately 25 ml sterile liquid GD media (containing 500 mg/L carbenicillin) before being transferred to sterile 100 µm mesh. Mesh was placed on sterile solid GD media containing 250 mg/L carbenicillin and the plates incubated at 16 hrs light / 8 hrs dark at 28 °C for 3 – 4 days before moving to sterile solid GD media containing 250mg/L carbenicillin and 5 mg/L hygromycin for 1 week. The mesh was then transferred to sterile solid GD media containing 250 mg/L carbenicillin, 8 mg/L hygromycin for 1 week and then solid GD media with 250 mg/L carbenicillin, 15 mg/L hygromycin for 1 week. The mesh harbouring the FECs was transferred to sterile MSN media (4.4 g/L Murashige and Skoog salts

with Gamborg's vitamins (Melford), 2% sucrose, 1 mg/L NAA) containing 250 mg/L carbenicillin and 15 mg/L hygromycin and incubated at 28 °C under 16 hrs light / 8 hrs dark for 10 days. Media was replenished every 10 days for as long as green embryos/cotyledons were appearing.

Regeneration of transgenic cotyledons: Hygromycin resistant embryos/cotyledons were transferred to sterile CMM (Cassava Maturation Media; 4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 2 µM CuSO₄, 2% sucrose, 0.3% Gelrite™, 0.1 mg/L BAP) for 1 week before transferring to sterile CEM (Cassava Elongation Media; 4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 2 µM CuSO₄, 2% sucrose, 0.3% Gelrite™, 0.4 mg/L BAP). Media was replaced every 2 weeks for approximately 4 – 5 weeks. Apical growth tips/shoots were isolated and placed in sterile CBM (Cassava Basal Media; 4.4 g/L Murashige and Skoog salts with Gamborg's vitamins (Melford), 2 µM CuSO₄, 2% sucrose, 0.3% Gelrite™) to establish plantlets.

Identification of transgenic lines: The apical growth tips of established plantlets were removed and planted in sterile CBM containing 10 mg/L hygromycin. After approximately 2 weeks transgenic plantlets should have formed a root system. No root growth is seen with wild-type and non-transgenic material.

2.2.12 Phenotypic analysis of transgenic plants

To assess germination efficiency, *Arabidopsis* seeds were surface sterilised and stratified at 4 °C for 3 days, then plated onto moist nitrocellulose membrane. Seeds were incubated at 22 °C for 4 days, after which time the number of germinated seeds and the total number of seeds was recorded. Three replicates were performed for each line. The primary root length was assayed by growing 20 plants of each line on solid MS media for 6 days, with plates incubated 5° from vertical. After the incubation, plates were photographed and the root lengths measured using ImageJ. Flowering time was recorded for 15 plants of each line when the first flower had reached stage 13 of development (Smyth *et al.*, 1990). Fertility was assessed in 5 DAF (days after fertilisation) siliques located approximately in the middle of the primary stem. Five siliques were opened manually for each line and the number of fertilised ovules and the total number of ovules recorded. This assay also provided the number of seeds per pod.

2.2.13 Plant stress treatments

Plate-based stress treatments were carried out by adding stress-inducing chemicals to warm MS media (approximately 45 °C) and pouring plates immediately. Seed was stratified at 4 °C for 3 days and twenty seeds of each line were germinated on the media and grown for 8-12 days, after which photographs were taken and the primary root lengths measured using ImageJ.

Soil-grown plants were stressed by adding stress-inducing chemicals during watering. Six control plants and six stressed plants received the same volume of water. The effect of the stress was measured by photographing plants and measuring the total photosynthetic area using ImageJ. Drought stress was induced by watering control and test plants equally up to 23 days after germination (DAG), then withholding water from the test plants for 7 days.

Wounding treatments were applied by piercing plant tissue with the end of sterile tweezers (pin-prick), severing tissue with sterile micro-scissors (slice) and squeezing tissue between the fingers (squeeze).

2.3 METHODS – RNA/DNA manipulation

2.3.1 RNA isolation and quantification

Leaf material was ground with liquid nitrogen in a sterile pestle and mortar. Total RNA was isolated from *Arabidopsis* leaf material using the SV Total RNA Isolation System (Promega). RNA was eluted from columns with 100 µl NFW. The concentration and quality of the RNA was determined by spectrophotometry readings at 230 nm, 260 nm and 280 nm. The concentration of RNA was calculated using the following equation:

$$\text{RNA (ng/}\mu\text{l)} = A_{260} \times \text{dilution factor} \times 40$$

The $A_{260}:A_{280}$ ratio assesses protein contamination of RNA samples and should ideally be greater than 1.8. The $A_{260}:A_{230}$ ratio assesses sample contamination by chemicals used in the RNA isolation procedure such as guanidine and phenol and should be greater than 2.0. After RNA quantification, 500 ng of each RNA sample was electrophoresed to confirm its integrity.

2.3.2 Semi-quantitative reverse transcription PCR (sqRT-PCR)

2.3.2.1 cDNA synthesis

The RevertAid™ First Strand cDNA Synthesis kit (Fermentas) was used to create cDNA from RNA template.

1 µg total RNA for each sample was combined with 1 µl random hexamer primer, 1 µl oligo(dT)₁₈ primer and DEPC-treated water to 12 µl in a 0.2 ml sterile thin-walled PCR tube. Samples were incubated at 70 °C for 5 mins and then chilled on ice. 4 µl 5x reaction buffer, 1 µl RiboLock™ RNase inhibitor (20 U/µl) and 2 µl 10 mM dNTPs were added in the order given. Samples were incubated at 25 °C for 5 mins. 1 µl RevertAid™ M-MuLV reverse transcriptase (200 U/µl) was added and the samples incubated at 25 °C for 10 mins, 42 °C for 60 mins and 70 °C for 10 mins. cDNA was stored at –20 °C.

2.3.2.2 sqPCR from cDNA

Each set of cDNA samples was standardised by amplifying the *Arabidopsis* house-keeping gene *ACTIN2* (At3g18780) using primer pair 19 (Table 2.3). This eliminated any differences in cDNA synthesis efficiency between samples.

sqPCRs were set up in sterile 0.2 ml thin-walled PCR tubes as follows: 1 µl cDNA, 2 µl 10x *Taq* DNA polymerase buffer, 2 µl dNTPs (1.25 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.2 µl *Taq* DNA polymerase (5 U/µl) and 12.8 µl SDW. *Taq* polymerase and its buffer were obtained from New England Biosciences and dNTPs were supplied by Promega. Reactions were cycled in a PTC-200 DNA Engine (MJ Research) as follows: 94 °C for 3 mins, 21x [94 °C for 45 secs, 55 °C for 45 secs, 72 °C for 45 secs], 72 °C for 10 mins. Products were electrophoresed on agarose gels.

sqPCRs were repeated with altered cDNA ratios between samples and/or an altered number of cycles until equal quantities of PCR product for samples in each set were achieved. Gene-specific sqPCRs were then carried out using the cDNA ratios established from the *ACTIN2* sqPCRs to determine relative abundances of target gene mRNA between samples. The PCR set-up was as above, with the number of cycles adjusted so that establishing differences between samples was possible (i.e. so that PCR products were not too intense after electrophoresis)

Primers used in sqPCR amplification were designed to distinguish amplification from genomic DNA (gDNA) and cDNA. Primers were designed either

to bridge exon-exon junctions so that amplification from gDNA was not possible or to span introns so that PCR products from gDNA were significantly larger than that from cDNA.

2.3.3 Plasmid DNA extraction

E. coli cultures were grown in 5 ml sterile liquid LB media with the appropriate antibiotic. After 16 hrs shaking (200 rpm) at 37 °C, cells were pelleted by spinning at 3,857 x g in a Universal 32R centrifuge (Hettich) and the supernatant discarded. Plasmid DNA was extracted from the cells using the QIAprep Spin Miniprep Kit (Qiagen). DNA was eluted in 55 µl SDW and the concentration and quality of the DNA was determined by spectrophotometry readings at 260 nm and 280 nm. The concentration of DNA was calculated using the following equation:

$$\text{DNA (ng/}\mu\text{l)} = A_{260} \times \text{dilution factor} \times 50$$

The $A_{260}:A_{280}$ ratio assesses protein contamination of DNA samples and should ideally be greater than 1.6.

2.3.4 gDNA extraction – *Arabidopsis*

The following was adapted from Edwards *et al* (1991).

A small piece of *Arabidopsis* leaf material was placed in a sterile 1.5 ml microfuge tube with 450 µl extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM sodium chloride and 25 mM EDTA) and a small amount of sterile sand. This was homogenised using a pillar drill with a sterile plastic pestle attachment. The resulting homogenate was centrifuged for 15 mins at 16,110 x g in a 5415D microcentrifuge (Eppendorf). 400 µl of supernatant was transferred to a clean, sterile microfuge tube and spun as before. 350 µl supernatant was transferred to a clean, sterile microfuge tube containing 350 µl 100% isopropanol and the mixture was incubated at room temperature for 15 mins. The centrifugation step was repeated and the supernatant discarded. The remaining pellet was washed three times with 200 µl 70% ethanol. Cleaned pellets were allowed to air dry for 10 mins and then resuspended in 100 µl SDW. gDNA samples were stored at 4 °C.

2.3.5 gDNA extraction – tobacco

The following was adapted from Dellaporta *et al* (1983).

DAY 1: Approximately 0.2 g of tobacco leaf was ground to a fine powder using a sterile pestle and mortar. The powder was combined with 750 µl extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM sodium chloride, 0.07% β-mercaptoethanol and 1% polyvinylpyrrolidone) prewarmed to 65 °C and 50 µl 20% SDS in a sterile 1.5 ml microfuge tube. The tube contents were mixed well and incubated at 65 °C for 10 mins in a water bath. After incubation, 250 µl ice-cold 5 M potassium acetate was added, tubes shaken vigorously and incubated on ice for 1 hr. Tubes were then centrifuged for 20 mins at 16,110 x g and 4 °C in a 5415D microcentrifuge (Eppendorf). The supernatant was filtered through sterile Miracloth (Calbiochem) into a clean, sterile microfuge tube containing 500 µl 100% isopropanol (cooled to –20 °C). After gentle inversion, tubes were incubated overnight at –20 °C to allow the gDNA to precipitate.

DAY 2: Tubes were centrifuged for 15 mins at 16,110 x g and 4 °C in a 5415D microcentrifuge (Eppendorf) and the supernatant discarded. The pellet was washed with 200 µl 70% ethanol and allowed to air dry before being resuspended in 70 µl 1x TE pH 8.0. RNase was added to a final concentration of 10 µg/ml and the samples incubated at 37 °C for 30 mins. DNA was precipitated by adding 7.5 µl 3 M sodium acetate pH 5.2 and 50 µl 100% isopropanol (cooled to –20 °C). After gentle inversion, tubes were incubated overnight at –20 °C.

DAY 3: Tubes were centrifuged for 15 mins at 16,110 x g and 4 °C in a 5415D microcentrifuge (Eppendorf) and the supernatant discarded. The pellet was washed twice with 50 µl 70% ethanol (cooled to –20 °C), air dried and resuspended in 50 µl SDW. Samples were stored at –20 °C.

2.3.6 Polymerase chain reaction (PCR)

2.3.6.1 PCR genotyping

PCR was used as a tool to genotype bacterial cultures (*E. coli* and *A. tumefaciens*) and plant material (*Arabidopsis* and tobacco). The presence/absence of a band was used to characterise the material tested for the presence of a target gene or a mutation. For bacterial genotyping, colonies to be tested were used to inoculate a solution containing 25 µl T0.1E pH 8.0 (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH

8.0) and 1 µl 0.7 mg/ml proteinase K (Roche) in a sterile thin-walled 0.2 ml PCR tube. Colonies were also replica plated onto LB agar plates with the appropriate antibiotic and grown for 16 hrs at 37 °C. The lysis mix was incubated at 55 °C for 15 mins, 80 °C for 15 mins and then on ice to terminate the reaction. 2 µl of the lysate was used as template in a genotyping PCR reaction. For genotyping of plant material, 2 µl of gDNA was used as template for the PCR step.

Genotyping PCRs were set up in sterile thin-walled 0.2 ml PCR tubes. The 20 µl reaction consisted of: 2 µl template (cell lysate/gDNA), 2 µl 10x *Taq* DNA polymerase buffer, 4 µl dNTPs (1.25 mM), 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.2 µl *Taq* DNA polymerase (5 U/µl) and 9.8 µl SDW. *Taq* polymerase and its buffer were obtained from New England Biosciences and dNTPs were supplied by Promega. Reactions were cycled in a PTC-200 DNA Engine (MJ Research) as follows: 94 °C for 3 mins, 35x [94 °C for 45 secs, X °C for 45 secs, 72 °C for Y mins], 72 °C for 10 mins. The annealing temperature (X °C) depends on the sequence and length of the primer, while the extension time (Y mins) was dependent on the length of the desired amplicon. The annealing temperature and extension times are therefore specific for each PCR and are given in Table 2.3. PCR products were electrophoresed on agarose gels.

2.3.6.2 PCR for cloning

A high fidelity polymerase was used when amplifying components of expression cassettes such as target genes or promoters. This was to ensure the template was replicated without error since base pair changes introduced through PCR can lead to truncated and non-functional coding sequences.

High-fidelity PCR extensions were performed with KOD DNA polymerase (Novagen) in sterile 0.2 ml thin-walled PCR tubes. Reactions consisted of: 10 – 100 ng DNA template, 5 µl 10x KOD DNA polymerase buffer, 2 µl 25 mM MgCl₂, 5 µl 2 mM dNTPs, 1.5 µl forward primer (10 µM), 1.5 µl reverse primer (10 µM), 1 µl KOD DNA polymerase and SDW to 50 µl. Reactions were cycled in a PTC-200 DNA Engine (MJ Research) as follows: 95 °C for 3 mins, 35x [95 °C for 30 secs, X °C for 30 secs, 70 °C for Y mins], 70 °C for 10 mins. The annealing temperature (X °C) and extension time (Y mins) for each PCR are given in Table 2.3.

2.3.7 Primers

Primers (Table 2.2) were designed using NetPrimer (Premier Biosoft) and were obtained from Sigma-Genosys. PCR conditions are given in Table 2.3.

Table 2.2: Primers used in this thesis.

Name	Sequence (5' → 3')	Primer T _m (°C)
Actin2F	GTA TGG TGA AGG CTG GAT TTG	57.22
Actin2R	CCC TCG TAG ATT GGC ACA GTG	60.43
apxF1	AAA GCA GGC TCA AAA ATG CCG AAG AAC TAC C	57.11
apxF2	GGA AAA ATG CCG AAG AAC TAC CC	63.38
APXgenericF	GST CCG WMT YGC ATG GCA CTC	65.19
APXgenericR	GAA KGC MTC TTC ATC WGC AGC	59.86
apxR1	AAG CTG GGT GTT ACG CCT CAG CAA ATC C	54.14
apxR2	GGA TTT GCT GAG GCG TAA GCT G	63.94
attB1	GGG GAC AAG TTT GTA CAA AAA AGC AGG CT	70.95
attB2	GGG GAC CAC TTT GTA CAA GAA AGC TGG GT	72.43
CAMBSeqF1	GGC TCG TAT GTT GTG TGG AAT TG	62.02
CAMBSeqR1	GCT ATG TAA TAT TTA CAC CAT AAC C	53.17
catF1	AAA AGC AGG CTC GGT CAT GGA TCC TTG CAA G	61.05
catF2	TTT GTC ATG GAT CCT TGC AAG TTC CGT CC	74.53
CATgenericF	CAC CAG AGA GGG VAA CTT TG	57.95
CATgenericR	ATC YTC AGG CCA GRT CTT K	54.37
CATintF1	GTC TCA CAT CCA AGA ATA CTG GAG	57.48
CATintR1	CAG CAA ACC AGT TAT CGA TGT TCC	62.86
catR1	GAA AGC TGG GTC TCA TAT ATT TGG CCT CAC G	53.39
catR2	TCA TCT TCA TAT ATT TGG CCT CAC GTT G	66.91
DeleteF1	GAC CTG CAG CCT CAG CAA AAG AAA AC	44.91
DeleteF2	CGA CTG CAG GTG AGT GAG GTG CGA G	44.73
DeleteF3	CGA CTG CAG CTC TCT TCT TAT CAA TC	33.44
DeleteR	TAC CAT GGC ATA TAA CAA GCA TGG	37.10
DESTSeqF1	CAT CAC TAA TGA CAG TTG CGG TGC	64.42
DESTSeqR1	GCA CAT ACA AAT GGA CGA ACG G	62.74
ETR1F	CGA AGT TGA TAA TGC TAT GTG TTT G	59.19
ETR1R	CTC CAC GGG ATG TTG ATG ACG AAG	67.51
GUSattfor	AAA AGC AGG CTC GAT GGT AGA TCT GAG GGT A	52.24
GUSattrev	GAA AGC TGG GTG TCA CAC GTG ATG GTG ATG G	56.75
GUSintR1	GAA GTT CGG CTT GTT ACG AAT GAC	62.29
JinF3	CAA GGT GAG AAC GAC CCG TCT	61.38
JinR3	CGC GTA GAA TCT TTG GTT TAG	55.29
JinR5	CTT GCT CTG AGC TGT TCT TGC G	62.86
M13F-20	GTA AAA CGA CGG CCA G	49.01
M13R	CAG GAA ACA GCT ATG AC	41.53
PATintR1	CAG TTG AAG TCA AAG TCA TAT CG	54.75
PATpstF	CCT CTG CAG TTG TAG TTA ATG CGT ATT AG	64.05

Table 2.2 (continued): Primers used in this thesis.

Name	Sequence (5' → 3')	Primer T _m (°C)
PATpmlR	<u>CCT CGT CAC GTG</u> CAT ATA ACA AGC ATG G	71.47
sodF1	AAA GCA GGC TCG ACA ATG GTG AAG GCC GTT G	65.77
sodF2	TCA CAT AGA ACA ATG GTG AAG GCC GTT GC	73.66
SODgenericF	CTT GGW GAY ACM ACW AAT GG	53.21
SODgenericR	CCY TTY CCR AGR TCA TCA GG	59.27
sodR1	AGA AAG CTG GGT CCT ATC CTT GCA AAC CA	48.74
sodR2	TGA ATC CCT GGG GAA TCA TTC TAT CCT TGC	73.73
transgeneR	CAC TTT GTA CAA GAA AGC TGG G	57.20

Restriction sites and **attB Gateway[®]** tags, underlined = linkers, blue = restriction enzyme recognition sites

Table 2.3: PCR conditions for each primer pair used in this thesis.

Primer Pair	Primers (F / R)	Template	Amplicon Length (bp)	Extension Time (secs)	Annealing Temperature (°C)
1	PATpstF PATpmlR	<i>StPAT</i> in pUC19	1,020	75	63
2	CAMBSeqF1 PATintR1	pCAM PAT preGW	729	85	55
3	apxF2 apxR2	<i>MecAPX2</i> in pBluescript	763	50	60
4	catF2 catR2	cDNA library	1,491	100	65
5	sodF2 sodR2	cDNA library	491	40	64
6	apxF1 apxR1	<i>MecAPX2</i> in pCR2.1-TOPO	775	50	54
7	catF1 catR1	<i>MecCAT1</i> In pCR2.1-TOPO	1,507	100	53
8	sodF1 sodR1	<i>MecSOD2</i> in pCR2.1-TOPO	487	40	53
9	<i>attB1</i> <i>attB2</i>	<i>MecAPX2</i>	817	60	60
		<i>MecCAT1</i>	1,543	105	60
		<i>MecSOD2</i>	523	45	60
		<i>GusPlus</i>	2,117	150	60
10	GUSattfor GUSattrev	<i>GusPlus</i> in pCAMBIA 1305.1	2,078	150	54
11	DeleteF1 DeleteR	<i>StPAT</i> promoter in pUC19	278	30	52

Table 2.3 (continued): PCR conditions for each primer pair used in this thesis.

Primer Pair	Primers (F / R)	Template	Amplicon Length (bp)	Extension Time (secs)	Annealing Temperature (°C)
12	DeleteF2 DeleteR	<i>StPAT</i> promoter in pUC19	487	40	52
13	DeleteF3 DeleteR	<i>StPAT</i> promoter in pUC19	779	50	52
14	DESTSeqF1 GUSintR1	<i>StPAT::GusP</i> Reporter	895	60	60
15	JinF3 JinR3	At1g32640	597	45	55
16	JinF3 JinR5	At1g32640	1,037	75	59
17	ETR1F ETR1R	At1g66340	690	45	57
18	CAMBSeqF1 CAMBSeqR1	<i>StPAT::GusP</i> reporter	1,302	90	55
		<i>D3::GusP</i> reporter	1,065	75	55
		<i>D2::GusP</i> reporter	773	60	55
		<i>D1::GusP</i> reporter	564	45	55
		– <i>StPAT::GusP</i> reporter	301	30	55
19	DESTSeqF1 apxR1	<i>StPAT::APX2</i>	952	65	54
20	APXgenericF transgeneR	<i>StPAT::APX2</i>	671	50	55
21	APXgenericF APXgenericR	Total APX in <i>Arabidopsis</i>	585	45	57
22	CATgenericF CATgenericR	Total CAT in <i>Arabidopsis</i>	509	40	54
23	SODgenericF SODgenericR	Total SOD in <i>Arabidopsis</i>	240	30	54
24	Actin2F Actin2R	<i>Actin2</i> (At3g18780)	538	45	55
25	DESTSeqF1 CATintR1	<i>StPAT::CAT1</i>	1,141	80	60
26	CATintF1 transgeneR	<i>StPAT::CAT1</i>	1,016	75	55
27	DESTSeqF1 sodR1	<i>StPAT::SOD2</i>	661	50	54
28	SODgenericF transgeneR	<i>StPAT::SOD2</i>	339	30	54

2.3.8 DNA restriction digestion and dephosphorylation of blunt ends

Restriction digestion of DNA was carried out with enzymes supplied by Promega, unless otherwise stated. The manufacturer's guidelines were followed. Approximately 1 µg plasmid DNA was digested and after incubation at 37 °C, reactions were stopped according to information supplied by the manufacturer. DNA fragments were analysed by agarose gel electrophoresis.

DNA fragments were dephosphorylated to prevent recircularisation using calf intestinal alkaline phosphatase (CIAP, Promega). The manufacturer's protocol was followed, with two rounds of incubation at 37 °C for 15 mins followed by 56 °C for 15 mins.

2.3.9 Agarose gel electrophoresis

DNA and RNA were visualised using agarose gel electrophoresis. 1x TBE buffer (90 mM Tris-borate, 2 mM EDTA) containing 1% agarose was boiled in a microwave until the agarose had melted. The solution was allowed to cool and ethidium bromide was added to a final concentration of 0.25 µg/µl. The solution was poured into a tank and allowed to set. DNA and RNA samples containing 1x loading buffer (5% glycerol, 0.05% bromophenol blue) were loaded onto gels immersed in 1x TBE buffer, along with 5 µl Quick-Load™ 1 kb ladder (New England Biolabs). Samples were electrophoresed for approximately 45 mins at 60 V. DNA/RNA was visualised using GDS 7500 UV transilluminator (UVP) and images were taken using Grab-IT 2.0 (Synoptics Ltd).

2.3.10 DNA clean-up and sequencing

To isolate DNA from agarose gels, bands were excised using a clean razor blade. DNA was purified from agarose using the Qiaquick gel extraction kit (Qiagen). DNA was eluted from columns with 40 µl SDW. To isolate DNA directly from PCR or digest reaction mixes, the Qiaquick PCR purification kit (Qiagen) was used. DNA was eluted from columns with 40 µl SDW. DNA sequencing was carried out by Cogenics or Eurofins MWG Operon.

2.3.11 Ligations

Ligations were carried out using T4 DNA Ligase (Promega). The protocol was followed, with reaction volumes doubled to 20 µl and the incubation performed at 16 °C for 16 hrs. An equal number of femtomoles of digested vector and insert were combined in each ligation. This was calculated using the following equation:

$$\text{ng vector/insert} = \text{fmoles required} \times \text{vector/insert size (bp)} \times 0.00066$$

Ligation reactions were stopped by incubating samples at 70 °C for 10 mins. 1 µl of ligation product was used to transform *E. coli* cells.

2.3.12 A-tailing and TOPO TA cloning®

TOPO TA cloning® permitted the ligation of a PCR product containing single 3' deoxyadenosine overhangs with the pCR®2.1-TOPO® vector containing single 3' deoxythymidine overhangs.

PCR products amplified with KOD DNA polymerase did not contain deoxyadenosine overhangs due to the proofreading capability of the enzyme and so were added in a separate step. 1 – 7 µl of PCR product was combined with 1 µl 10x Taq DNA polymerase buffer, 1 µl 2 mM dATP, 1 µl Taq DNA polymerase and SDW to 10 µl. The reaction was incubated at 70 °C for 20 mins. 1 µl of this solution was carried forward into the cloning procedure using the TOPO TA Cloning® kit (Invitrogen). The kit protocol was followed and 2 µl was subsequently used to transform *E. coli* cells.

2.3.13 Gateway® cloning

The Gateway® cloning procedure consists of two steps – the BP reaction and the LR reaction. The system is based on the site-specific recombination system of bacteriophage lambda. Gateway® technology uses modified versions of *att* sites to permit the transfer of DNA sequences between plasmids (Hartley *et al.*, 2000). *attB* sites recombine with *attP* sites in the presence of BP Clonase™ II (Figure 2.1A) and *attL* sites recombine with *attR* sites in the presence of LR Clonase™ II (Figure 2.1B). The process is also directional as a result of the complementarity between pairs of *att* sites. The addition of *attB* sites to the ends of PCR products allows them to recombine with an *attP* site-containing plasmid (pDONR™/Zeo).

This recombination event converts the *attP* sites in the plasmid to *attL* sites, permitting further recombination with an *attR* site-containing destination/expression plasmid (pCAM PAT GW). The process is split into two steps since the direct addition of lengthy *attL* sites to DNA sequences is impractical through conventional PCR.

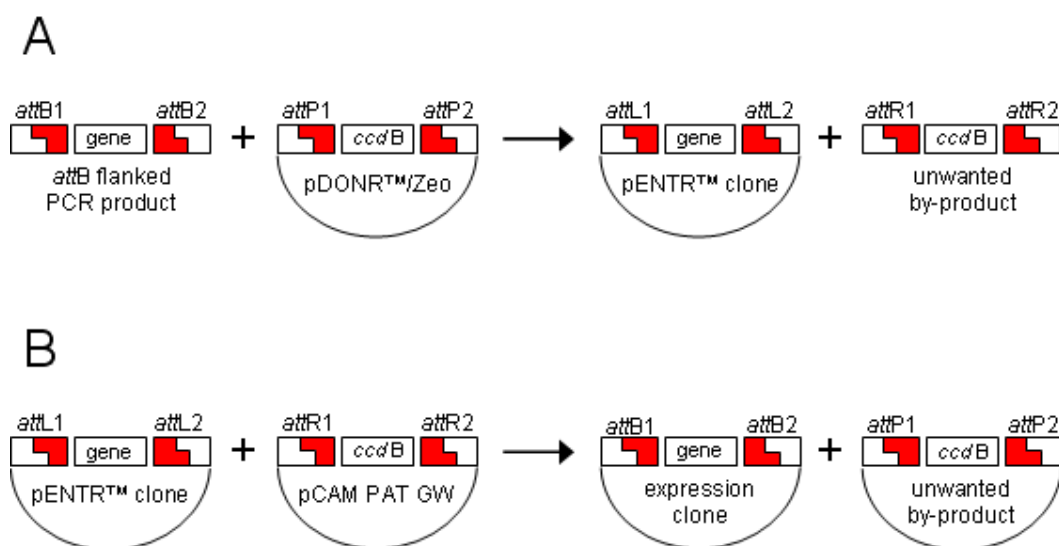


Figure 2.1: The Gateway® recombination reactions. (A) The BP reaction catalysed by BP Clonase™ II and (B) the LR reaction catalysed by LR Clonase™ II. Reproduced from the Gateway® Technology with Clonase™ II manual version A, June 2004 (Invitrogen).

attB sites were added to DNA sequences in two PCR steps due to their length. The first PCR utilised primers specific to the target sequence with a tail consisting of half the relevant *attB* site. The second PCR made use of primers specific to the whole *attB* site (*attB1* and *attB2* primers) and used the product from the first PCR as template.

Gateway® recombination reactions were carried out using the BP and LR Clonase™ enzyme mixes (Invitrogen) by following the manufacturer's protocols. The pDONR™/Zeo plasmid was obtained from Invitrogen. Conversion of pCAMBIA 1305.1 to a Gateway®-compatible plasmid was achieved using the Gateway® Vector Conversion System (Invitrogen). The creation of the Gateway®-compatible destination/expression plasmid (pCAM PAT GW) and the molecular cloning of target genes into pCAM PAT GW are described in Chapter 3.

2.4 METHODS – Biochemical assays

2.4.1 Total protein extraction for enzyme assays

Cassava tubers were washed and the peel removed. Tissue was grated into a sterile pestle and mortar containing liquid nitrogen and ground to a fine powder. An equal volume of ice-cold 50 mM HEPES buffer pH 7.0, 2 mM sodium metabisulphite was added and the mortar contents mixed. The homogenate was strained through a layer of sterile Miracloth (Calbiochem) into a 50 ml centrifuge tube. Samples were spun for 30 mins at 10,000 x g and 4 °C in an Avanti J-26 XP centrifuge with JA-25.50 rotor (Beckman Coulter). The supernatant was transferred to a clean, sterile tube and stored at –20 °C.

Arabidopsis plants grown on MS media plates were snap-frozen in liquid nitrogen. The root system was then detached from the rest of the plant by breaking just below the hypocotyl and transferred straight to liquid nitrogen. Samples were then processed in the same way as cassava storage root samples.

2.4.2 Bradford assay

The Bradford assay was used to quantify the total protein in root extracts prepared according to the method in Section 2.4.1 and was based on the protocol of Bradford *et al* (1976). 50 µl of root extract was combined with 1.5 ml Bradford Reagent and incubated at room temperature in the dark for 35 mins. The A_{595} was then measured against a blank of Bradford Reagent. A_{595} readings were converted to total protein content (mg/ml) by comparing to a standard curve generated using known concentrations of BSA (New England Biolabs).

2.4.3 Ascorbate peroxidase (APX) assay

APX enzyme activity of root extracts was assayed using a spectrophotometric method to measure the rate of guaiacol oxidation (tetraguaiacol formation) (Chance & Maehly, 1955). This assay actually assesses the activity of both APX and guaiacol peroxidase since both enzymes can use guaiacol as an electron donor (Mehlhorn *et al.*, 1996).

950 µl APX assay buffer (50 mM sodium acetate, 15 mM guaiacol) was placed in a plastic cuvette. 10 µl 50 mM HEPES buffer pH 7.0, 2 mM sodium metabisulphite was added and the sample loaded into a Cary 50 recording spectrophotometer (Varian) with the wavelength set at A_{470} . The instrument was

set to take a reading every 0.5 secs and to maintain the temperature at 30 °C. The 10 min run was started and 40 µl 240 mM H₂O₂ added. During the run, a line was plotted showing the relationship between time elapsed and the A₄₇₀. The change in A₄₇₀ over time was calculated at the end of the run by measuring the gradient of the line and represented the rate of reaction. This was repeated with pure horseradish peroxidase (HPX) enzyme to generate a standard curve. Root extracts were then tested and A₄₇₀ readings converted to units of APX via the standard curve. Two technical replicates were carried out for each standard and experimental sample. Samples were standardised using the total protein content to account for differences in protein extraction efficiency between samples.

2.4.4 Catalase (CAT) assay

CAT enzyme activity was assayed by following the degradation of hydrogen peroxide (H₂O₂) over time (Chance & Maehly, 1955).

940 µl CAT assay buffer (100 mM sodium phosphate buffer pH 6.0) was placed in a quartz cuvette. 10 µl 50 mM HEPES buffer pH 7.0, 2 mM sodium metabisulphite was added and the sample loaded into a Cary 50 recording spectrophotometer (Varian) with the wavelength set at A₂₄₀. The instrument was set to take a reading every 0.5 secs and to maintain the temperature at 30 °C. The 8 min run was started and 50 µl 240 mM H₂O₂ added. During the run, a line was plotted showing the relationship between time elapsed and the A₂₄₀. The change in A₂₄₀ over time was calculated at the end of the run by measuring the gradient of the line and represented the rate of reaction. This was repeated with pure CAT enzyme to generate a standard curve. Root extracts were then tested and A₂₄₀ readings converted to units of CAT via the standard curve. Two technical replicates were carried out for each standard and experimental sample. Samples were standardised using the total protein content to account for differences in protein extraction efficiency between samples.

2.4.5 Superoxide dismutase (SOD) assay

SOD enzyme activity of root extracts was assayed using a spectrophotometric method, which measured the photoreduction of nitro blue tetrazolium (NBT) (Beauchamp & Fridovich, 1971).

900 µl of SOD assay buffer (50 mM potassium phosphate buffer pH 7.8, 100 µM EDTA and 13 mM methionine) was placed into a plastic cuvette with 10 µl 50 mM HEPES buffer pH 7.0, 2 mM sodium metabisulphite, 10 µl 7.5 mM nitro blue tetrazolium and 80 µl 25 mM riboflavin. Cuvette contents were mixed and placed in the dark for 4 mins. The A_{560} of this sample was recorded as the blank. The above was repeated, this time incubating in a light box fitted with a 15 W fluorescent bulb (Prolite) for 2 mins, then in the dark for 2 mins and the A_{560} taken. The A_{560} should be maximal in a reaction without SOD. This was then repeated using known amounts of pure SOD enzyme in place of the HEPES buffer to generate a standard curve. Root extracts were then tested and A_{560} readings converted to units of SOD via the standard curve. Two technical replicates were carried out for each standard and experimental sample. Samples were standardised using the total protein content to account for differences in protein extraction efficiency between samples.

2.4.6 Histochemical staining for β -glucuronidase (GusP)

Histochemical GusP assays were carried out via a method adapted from Kosugi *et al* (1990). *Arabidopsis* or tobacco tissue was incubated in GusP buffer (100 mM potassium phosphate buffer pH 7.0, 1 mg/ml x-gluc, 0.1% Triton X-100, 3 mM potassium ferricyanide and 3 mM potassium ferrocyanide) for 1 hr at 37 °C. Plant tissues were washed in SDW to stop further GusP staining and then incubated in 70% ethanol until all tissue pigments (except GusP) were removed. Stained tissue samples were viewed under a SMZ1500 dissecting microscope (Nikon) and photographs taken using a Digital Sight DS-U1 colour camera (Nikon). Images were processed using Photoshop Elements (Adobe).

2.5 METHODS – Statistical analyses

All statistical analyses were carried out using Minitab 15.

Prior to analysis of continuous data, an Anderson-Darling test was carried out to ensure it followed a normal distribution, followed by a Levene's test to check for equal variances. A one-way ANOVA analysis with a Tukey's comparison (family error rate 5) was performed where necessary. Student's *t*-tests (2 sample, 2-tailed) were calculated with equal variances assumed. Data not following a normal

distribution was analysed using the Mann-Whitney U -test, with the p-value adjusted for ties.

3 PLASMID DESIGN AND CREATION

3.1 Introduction

3.1.1 Cloning needs

This research project is largely focused on the production and analysis of *Arabidopsis* and cassava plants over-expressing target genes in a root- or storage root-specific manner to assess their potential to modulate oxidative stress responses. The *StPAT* promoter was chosen (as described in Chapter 4) to bring about root- or storage root-specific expression of the antioxidant cassava genes *ASCORBATE PEROXIDASE 2* (*MecAPX2*, GenBank AY973622), *CATALASE 1* (*MecCAT1*, GenBank AF170272) and *SUPEROXIDE DISMUTASE 2* (*MecSOD2*, GenBank AY642137). An efficient transformation system is required to enable multiple successful transformation events. An essential part of the transformation process is an efficient and simple cloning system.

3.1.2 Choosing a suitable expression plasmid

Ideally, a suitable expression plasmid for this investigation should contain: a plant selectable marker that permits fast and simple screening of plants; the *StPAT* promoter to confer root- or storage root-specific transgene expression; and suitable sites for the efficient insertion of transgenes in front of the root-specific promoter. Unfortunately, such a plasmid was not available in the public domain and so an existing plasmid with some of the appropriate features was obtained and modified so that it was suitable. pCambia 1305.1 (Figure 3.1, GenBank AF354045) was chosen as the expression plasmid since it contains the *hpt II* gene (hygromycin B resistance) as the plant selectable marker, which enables simple and quick selection of *Arabidopsis*, tobacco and cassava plants (Harrison *et al.*, 2006). The promoter driving transgene expression is the constitutive CaMV35S promoter and is therefore unsuitable for this study; although restriction enzyme recognition sites exist that allow the insertion of the *StPAT* promoter in its place. Restriction sites are also present that would allow restriction cloning of transgenes in front of the promoter, although restriction cloning is far from efficient. The CaMV35S promoter is currently driving the expression of the *GusPlus* reporter gene. This plasmid is freely available to the research community.

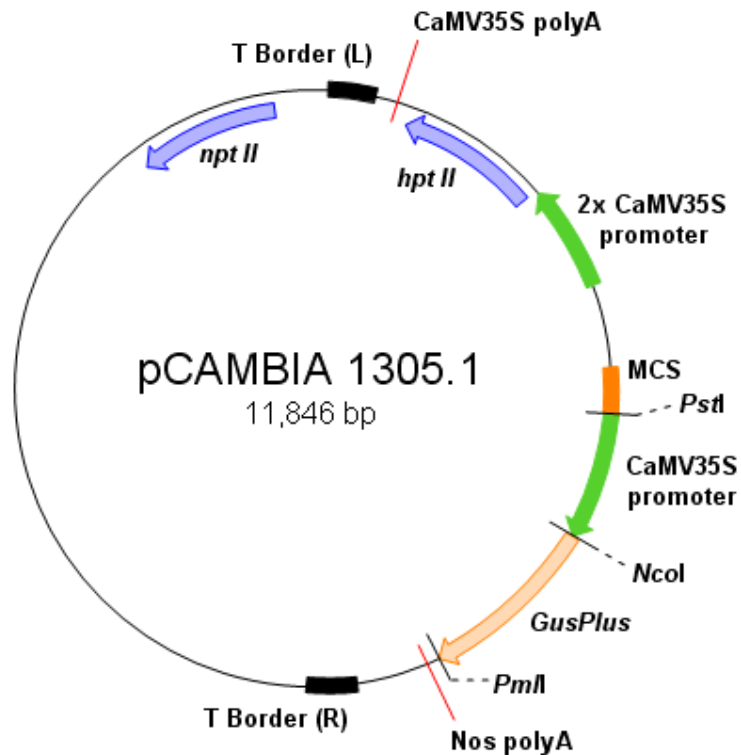


Figure 3.1: The pCambia 1305.1 expression plasmid. The plasmid backbone carries the *npt II* gene for resistance to kanamycin (bacteria). The T-DNA region carries the *hpt II* gene for resistance to hygromycin B (plants) and the *GusPlus* reporter gene. The reporter gene is constitutively driven by a CaMV35S promoter. MCS = multiple cloning site.

3.1.3 Choosing an appropriate cloning system

A good cloning system should be simple to execute and highly efficient. The Gateway[®] cloning system, originally developed by Life Technologies (Hartley *et al.*, 2000) but now distributed by Invitrogen, meets these requirements. Gateway[®] technology is based on the site-specific recombination properties of bacteriophage lambda and so is not affected by some of the limitations of restriction enzyme cloning (Park & LaBaer, 2006). The efficiency of the Gateway[®] system is 90-99%, far higher than restriction enzyme cloning, and does not depend on the presence or absence of specific restriction enzyme recognition sites at certain locations in plasmids. The recombination step of 1 hr is considerably faster than the process of conventional cloning (restriction digest – agarose gel electrophoresis – DNA clean-up – ligation). Additionally, once a Gateway[®]-compatible expression plasmid has been created or purchased, the system is high-throughput since target genes can readily be amplified and recombined into the expression plasmid

using one simple and robust method (Karimi *et al.*, 2007). The Gateway[®] cloning system is now widely used by the research community and it has been stated that the system has become “a universal standard in genomic research” (Watson *et al.*, 2005). In the five years since its introduction, the number of citations using Gateway[®] technology rose from 6 to 686 (Invitrogen, 2009). As pCAMBIA 1305.1 needed modifying to replace the CaMV35S promoter with the *StPAT* promoter, it was decided to make the plasmid Gateway[®]-compatible at the same time in a two step process.

The terminology used in this chapter is that of the Gateway[®] technology. pDONR[™] refers to an empty intermediate Gateway[®] plasmid, while pENTR[™] refers to a pDONR[™] plasmid into which a target gene has been recombined and may be followed by the target gene abbreviation. A binary plasmid containing a promoter driving a target gene is referred to either as an expression plasmid or pDEST[™] followed by the target gene abbreviation.

3.1.4 Research Aims

This chapter describes the methods used to integrate the *StPAT* promoter and transgenes into an expression vector format. Gateway[®] technology was employed to generate a simple and robust system for cloning the target genes. An existing expression vector, pCAMBIA 1305.1, was modified to make it compatible with the Gateway[®] system. A reporter expression vector was also created, as well as reporter expression vectors containing a series of *StPAT* promoter deletions for functional analysis of the *StPAT* promoter.

It should be stated here that information in this chapter is given to demonstrate how the expression plasmids were created and how the Gateway[®] system was used to meet the cloning needs of this project, rather than to demonstrate success at each step during cloning. Individual gel images and sequence data are not given here. Where cloning procedures have been verified through restriction digest, PCR genotyping or sequencing, it can be assumed that correct samples were identified.

3.2 Results

3.2.1 Modification of pCAMBIA 1305.1 – Step 1

The first stage of the modification of pCAMBIA 1305.1 involved the removal of the CaMV35S promoter and the *GusPlus* gene, followed by insertion of the *StPAT* promoter. The removal of the *CaMV35S::GusPlus* cassette was achieved by digesting pCAMBIA 1305.1 plasmid DNA with the *Pst*I and *Pml*I restriction enzymes (Figure 3.2A and 3.2B). An overnight incubation was performed to ensure the DNA had been cut to completion. Digested DNA was electrophoresed and the presence of the correct banding pattern was confirmed (9,028 bp and 2,818 bp). The desired fragment (9,028 bp) was gel purified from the agarose gel. The *StPAT* promoter was amplified from pUC19 using primers with *Pst*I (5') and *Pml*I (3') linkers added (primer pair 1). After purification, clean PCR product was subjected to an overnight restriction digest with *Pst*I and *Pml*I (Figure 3.2C). The digested pCAMBIA 1305.1 plasmid and the digested *StPAT* promoter were fused via a ligation reaction to create pCAM PAT preGW (Figure 3.2D). 1 µl of ligation mix was used to transform *E. coli* One Shot® TOP10 chemically competent cells. Transformed cells were plated onto sterile LB agar plates with 50 µg/ml kanamycin to select for the promoter-vector fusion. Plasmid DNA was extracted from resulting colonies and checked via PCR genotyping for the presence of pCAMBIA 1305.1 backbone and *StPAT* promoter sequence (primer pair 2). Samples identified as negative were either a result of recircularised plasmid (although the digested plasmid had overhangs that were not complimentary) or false positives from the kanamycin screen. Samples that were genotyped as positive were sequenced (CAMBseqF1 and DESTSeqR1 primers) to ensure the *StPAT* promoter sequence was correct and integration of the promoter had occurred as had been expected.

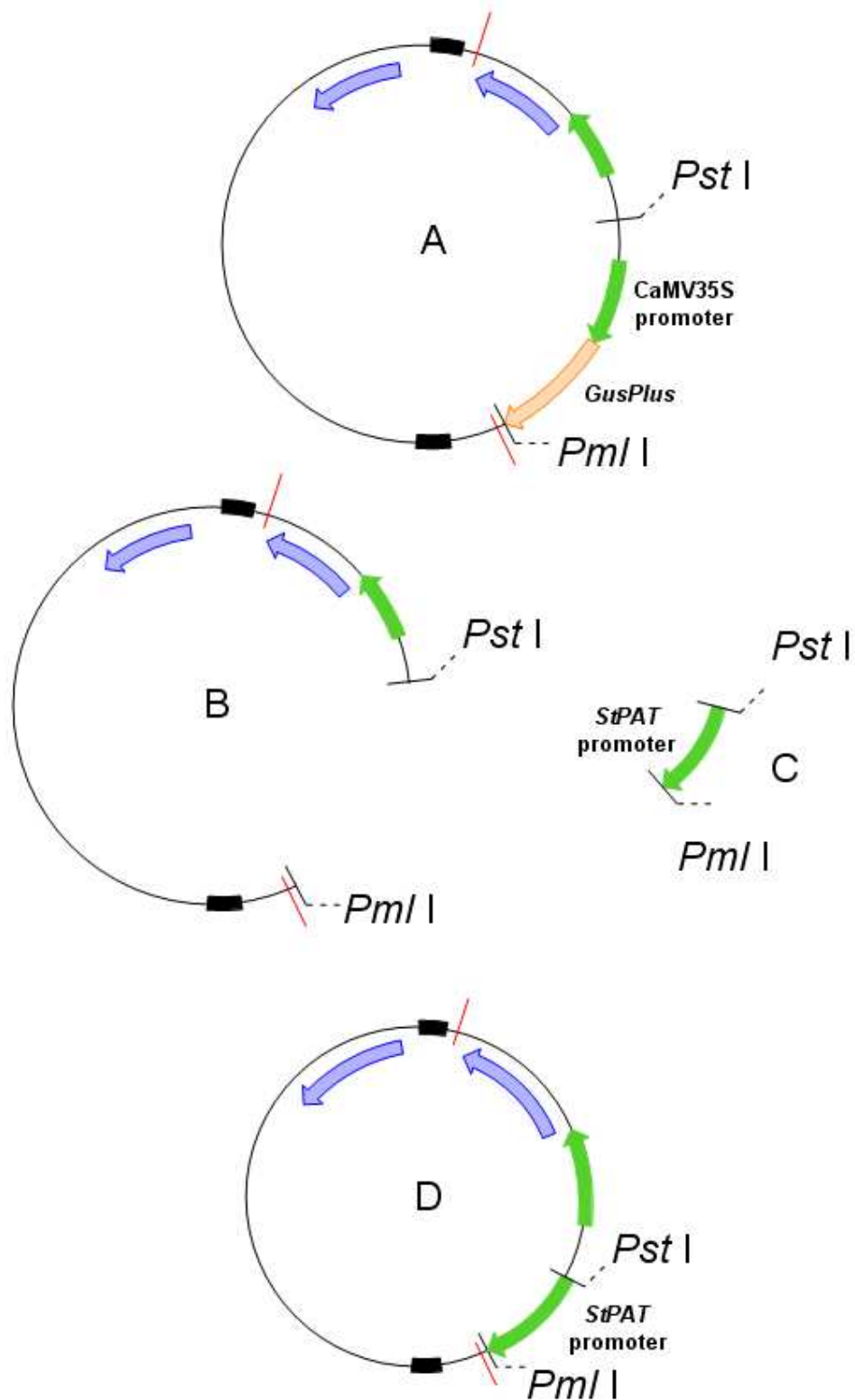


Figure 3.2: The conversion of pCambia 1305.1 into a Gateway®-compatible expression vector – Step 1. pCambia 1305.1 (A) was digested with *Pst*I and *Pml*I to remove the CaMV35S promoter and the *GusPlus* reporter gene (B). The *StPAT* promoter, containing *Pst*I (5') and *Pml*I (3') linkers (C) was ligated into the digested plasmid to create pCAM PAT preGW (D). Red lines are terminator/polyA sites.

3.2.2 Modification of pCAMBIA 1305.1 – Step 2

The second step in modifying pCAMBIA 1305.1 was the insertion of the Gateway[®] cassette. This cassette contained *attR* sites flanking the *ccdB* and *Cm^R* genes and would allow the pCAMBIA-based expression plasmid to recombine with a pENTR[™] plasmid carrying *attL* sites flanking a target gene. The reading frame A (RfA) cassette was used, although maintenance of the correct reading frame was not essential since N- or C-terminal fusions were not added to target gene sequences.

The Gateway[®] cassette is provided as a blunt-ended DNA fragment and must be inserted into linearised pCAM PAT preGW plasmid DNA directly downstream of the 3' end of the *StPAT* promoter. The ligation of the pCAMBIA 1305.1 plasmid backbone and the *StPAT* promoter described above (Section 3.2.1) resulted in the reformation of both the *PstI* and *PmlI* restriction enzyme recognition sites at the points of ligation. Importantly, the *PmlI* site was located directly downstream of the 3' end of the *StPAT* promoter and *PmlI* leaves blunt ends at sites where it cuts DNA. These properties of *PmlI* made it the ideal candidate for linearising pCAM PAT preGW, thereby allowing the insertion of the blunt-ended Gateway[®] cassette.

pCAM PAT preGW plasmid DNA was digested overnight with *PmlI* (Figure 3.3A). The resulting linearised pCAM PAT preGW plasmid was treated with CIAP to prevent recircularisation of the plasmid. The linearised, CIAP-treated plasmid (Figure 3.3B) and the provided blunt-ended Gateway[®] cassette (Figure 3.3C) were ligated together. The product of this ligation, pCAM PAT GW (Figure 3.3D) was used to transform *E. coli* DB3.1[™] competent cells since these cells were resistant to the negative effects of the *ccdB* gene. Colonies were analysed for the presence of the Gateway[®] cassette by extracting plasmid DNA and digesting it with *BsrGI*, an enzyme recognition site specific to Gateway[®]-related sequences. Digest products were analysed by electrophoresis and compared to the expected banding pattern (10,066 bp, 1,283 bp and 402 bp). The blunt end ligation of the Gateway[®] cassette was not directional and so plasmid DNA was also sequenced with DESTSeqF1, which bound 137 bp upstream of the insertion site of the Gateway[®] cassette and read into it. Samples were identified that carried the Gateway[®] cassette in the correct orientation (i.e. 5': *StPAT* promoter → *attR1* → *Cm^R* → *ccdB* → *attR2*: 3').

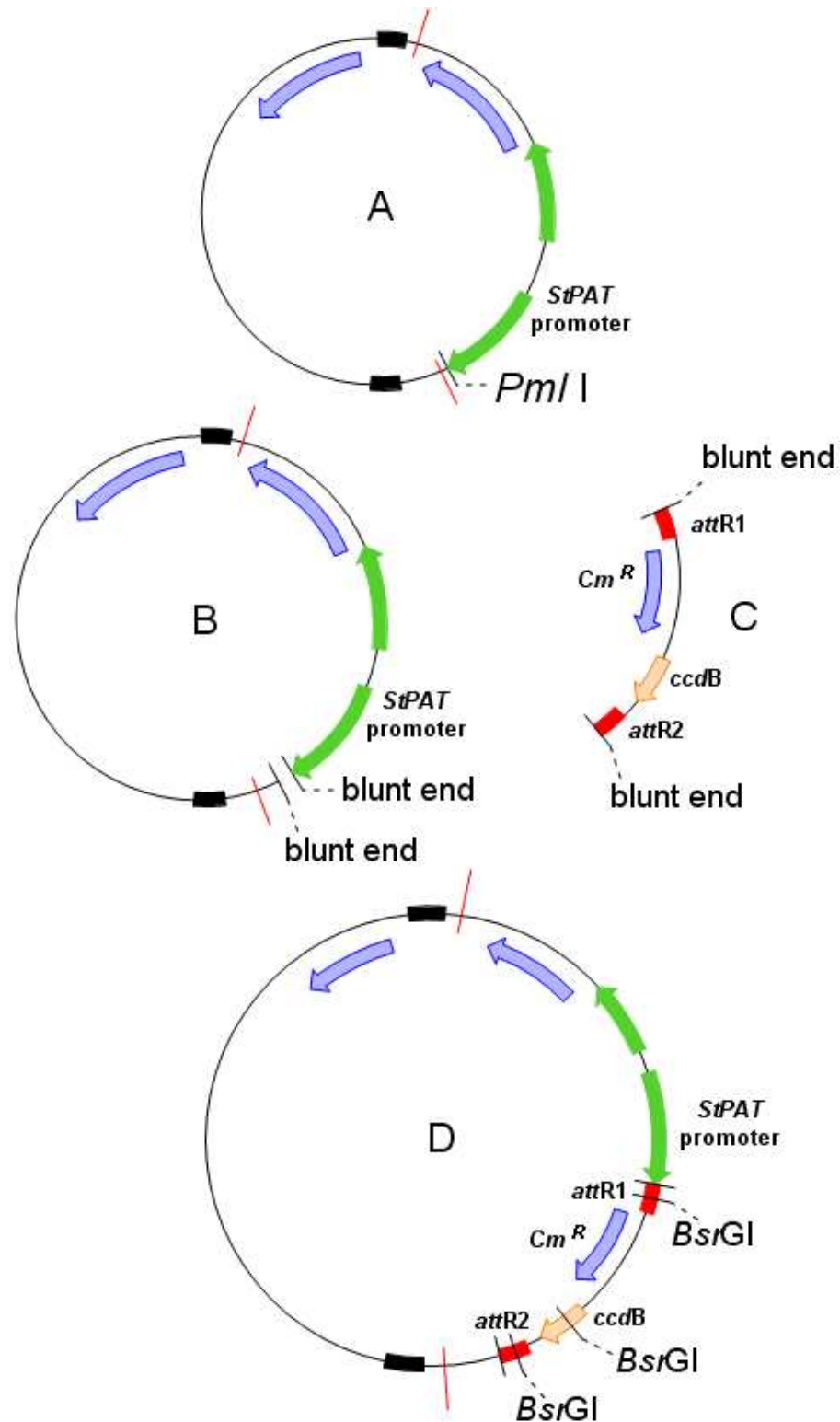


Figure 3.3: The conversion of pCAMBIA 1305.1 into a Gateway[®]-compatible expression vector – Step 2. pCAM PAT preGW (A) was digested with *Pml*I to linearise the plasmid leaving blunt ends (B). The Gateway[®] cassette (C) was ligated in to create the Gateway[®]-compatible expression vector, pCAM PAT GW (D). Red lines are terminator/polyA sites.

3.2.3 Isolation of target genes

The three target genes (*MecAPX2*, *MecCAT1* and *MecSOD2*) had all previously been isolated from cassava and the cDNA sequences determined (Reilly *et al.*, 2001; Reilly *et al.*, 2004; Shin *et al.*, 2005). Furthermore, the *MecAPX2* cDNA had previously been amplified from an existing cDNA library and cloned into a pBluescript vector. As a result, it was possible to amplify *MecAPX2* (primer pair 3) directly from the pBluescript clone. Since full-length clones of *MecCAT1* and *MecSOD2* were not available, these cDNA sequences were amplified from an existing cDNA library created in λ gt10 bacteriophage. The library was created using mRNA isolated from cassava storage roots. A 2 μ l aliquot of neat cDNA library was used as template for the amplification of *MecCAT1* (primer pair 4) and *MecSOD2* (primer pair 5). Successful amplification of target cDNAs was followed by A-tailing and then cloning into the pCR[®]2.1-TOPO[®] plasmid, which contained T-overhangs. The resulting plasmids were used to transform *E. coli* DH5 α , which were then selected on LB agar media with 50 μ g/ml kanamycin. Colonies were genotyped via PCR with the same primer pairs that were used to amplify the genes and then sequenced (M13F-20 and M13R primers) to confirm target gene sequence integrity.

3.2.4 Addition of *attB* Gateway[®] sites to target genes

At 29 bp, the *attB* sites were considered too long to incorporate onto the ends of primers and so two separate PCRs were carried out. The first PCR used primers consisting of target gene sequence and a section of *attB* site sequence. The second PCR utilised primers consisting of the full *attB* site sequences, using the product from the first PCR as template. *attB1* sites were added at the 5' end of target genes with forward primers and *attB2* sites were added at the 3' ends using reverse primers.

When designing *attB1*-containing forward primers for the first round of PCR, the target genes should be immediately preceded by their native Kozak sequences (Kozak, 1987) to confer maximal expression. Additionally, the sequence upstream of the target genes (including the *attB1* site and the sequence between *attB1* and the translational start site) should not include a STOP codon (TAA, TGA or TAG) to ensure transcription of target genes. As an example, the forward primer used in the first PCR adding *attB1* sequence to *MecAPX2* is shown below (Figure 3.4). The five base pairs between *attB1* and *MecAPX2* sequence are used to

incorporate the native Kozak sequence. The actual sequence of *MecAPX2* upstream of the translational start site is GAAAA, but here the G was replaced with a C to avoid introducing a premature STOP codon. A single base pair was added between the *attB2* sequence and the STOP codon of the target genes in the reverse primer. This base pair was chosen based on the quality rating of the primer when analysed with NetPrimer.

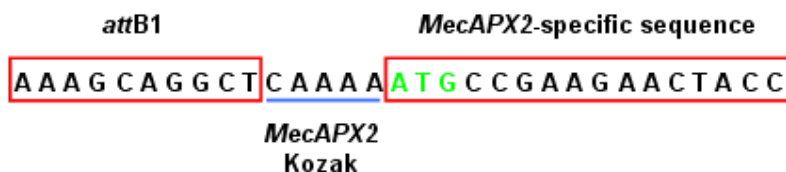


Figure 3.4: The apxF2 primer. This primer added a section of the *attB1* site to *MecAPX2*. The translational start site of *MecAPX2* is highlighted in green, base pairs added to introduce the native Kozak sequence of *MecAPX2* are underlined in blue.

attB-containing primers (*MecAPX2* – primer pair 6; *MecCAT1* – primer pair 7; *MecSOD2* – primer pair 8) were used to amplify the target genes from pCR[®]2.1-TOPO[®] (Figure 3.5A). This resulted in target genes with a portion of *attB* sequence (Figure 3.5B). These were used as templates in a second PCR to add the full *attB* sequences using primer pair 9 (Figure 3.5C). The final PCR product was used directly in the BP reaction.

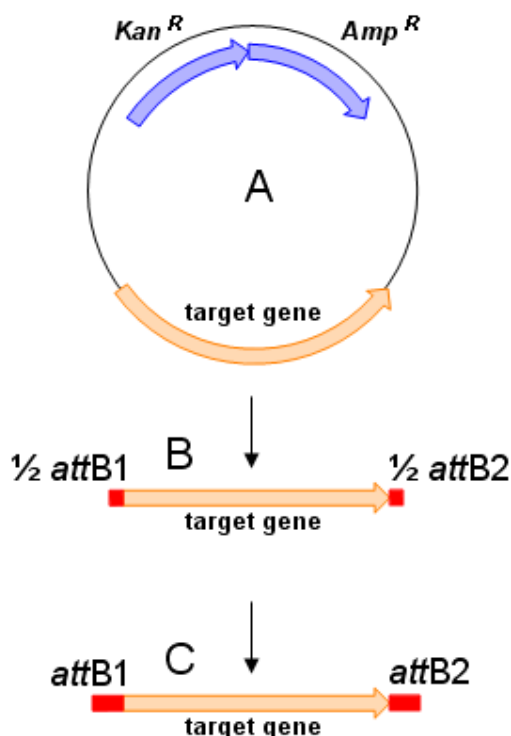


Figure 3.5: Amplification of target genes to incorporate *attB* sites. Target genes were amplified from pCR[®]2.1-TOPO[®] (A) via PCR, adding half-length *attB* sites (B). A second round of PCR added the full-length *attB* sites to the target genes (C).

3.2.5 The BP reaction

The intermediate Gateway[®] plasmid, pDONR[™]/Zeo (Figure 3.6A), was recombined with *attB*-containing target genes (Figure 3.6B) in the BP reaction. This reaction should generate a pENTR[™] plasmid containing a target gene (Figure 3.6C) as well as the Gateway[®] cassette as an unwanted by-product (Figure 3.6D). An equal number of femtomoles of *attB*-tagged DNA and *attP*-containing pDONR[™]/Zeo were combined in the BP reaction, which was catalysed by BP Clonase[™] II. After a 1 hr incubation at 25 °C, reactions were stopped using the provided proteinase K. Recombined DNA was used to transform *E. coli* One Shot[®] TOP10 cells, which were then selected on LB agar media with 50 µg/ml Zeocin[™]. LB liquid media cultures with 50 µg/ml Zeocin[™] were inoculated with individual colonies. Plasmid DNA was extracted from cultures and screened by digesting with *Bsr*GI. Colonies containing pDONR[™]/Zeo plasmid that had not recombined should not survive due to the negative selective pressure imposed by

the *ccdB* gene. However, if colonies did persist on the selection media, three bands would be seen after the *Bsr*GI plasmid DNA digest whereas successfully recombined pENTR™ plasmid DNA would yield two bands. Plasmid DNA with the correct banding pattern was sequenced (M13F-20 and M13R universal primers) to confirm the integrity of the target gene sequence. Three plasmids were generated using the BP reaction: pENTR™ *MecAPX2*, pENTR™ *MecCAT1* and pENTR™ *MecSOD2*.

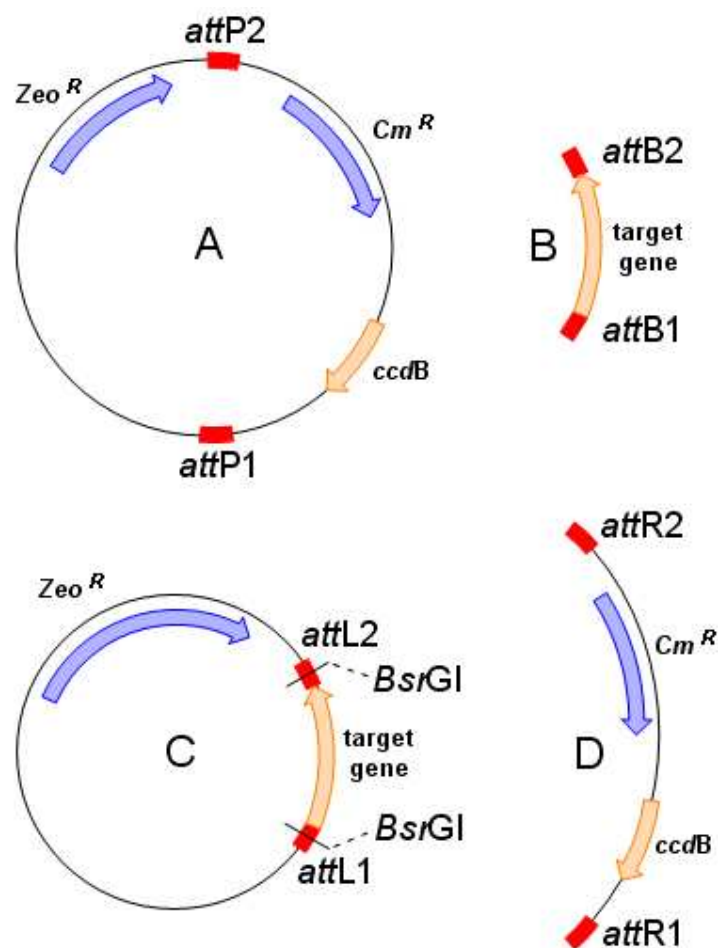


Figure 3.6: The BP reaction – recombination of target genes into the intermediate Gateway® plasmid, pDONR™/Zeo. Complimentary *att* sites on pDONR™/Zeo (A) and target genes (B) permit recombination via BP Clonase™ II. Recombination results in a pENTR™ plasmid harbouring the target gene (C) and an unwanted by-product, the Gateway® cassette (D).

3.2.6 The LR reaction

A pENTR™ plasmid containing a target gene was recombined with the Gateway®-ready expression plasmid, pCAM PAT GW. *attL* sites on the pENTR™ plasmid recombined with *attR* sites on pCAM PAT GW, allowing the directional insertion of the target gene into the expression plasmid in front of the *StPAT* promoter.

An equal number of femtomoles of pENTR™ plasmid (Figure 3.7A) and pCAM PAT GW plasmid (Figure 3.7B) were combined and 2 µl LR Clonase™ II added. The products of this reaction were an expression plasmid (Figure 3.7C) and a smaller unwanted plasmid (Figure 3.7D). The reactions were incubated at 25 °C for 1 hr and then stopped using the provided proteinase K. Recombined DNA was used to transform *E. coli* One Shot® TOP10 cells, which were subsequently selected on LB agar media with 50 µg/ml kanamycin. Colonies were used to inoculate liquid LB agar media with 50 µg/ml kanamycin and plasmid DNA was extracted from the resulting cultures and screened via a *BsrGI* restriction digest. As in the BP reaction, colonies containing pCAM PAT GW plasmid that had not recombined should not survive due to the negative selective pressure imposed by the *ccdB* gene. However, if colonies did persist on the selection media, three bands would be seen after the plasmid DNA digest whereas successfully recombined expression plasmid DNA would yield two bands. Additionally, colonies containing the intermediate pENTR™ plasmids should not survive since they confer resistance to Zeocin™ and not to kanamycin. As PCR had not been carried out on any of the target genes since the addition of *attB* sites (Section 3.2.4), no sequence errors should be present in the target gene. However, plasmid DNA with the correct banding pattern was sequenced over the recombination boundaries (DESTSeqF1 and DESTSeqR1 primers) to confirm the successful integration of the target genes. Three plasmids were generated using the LR reaction: pDEST™ *MecAPX2*, pDEST™ *MecCAT1* and pDEST™ *MecSOD2*.

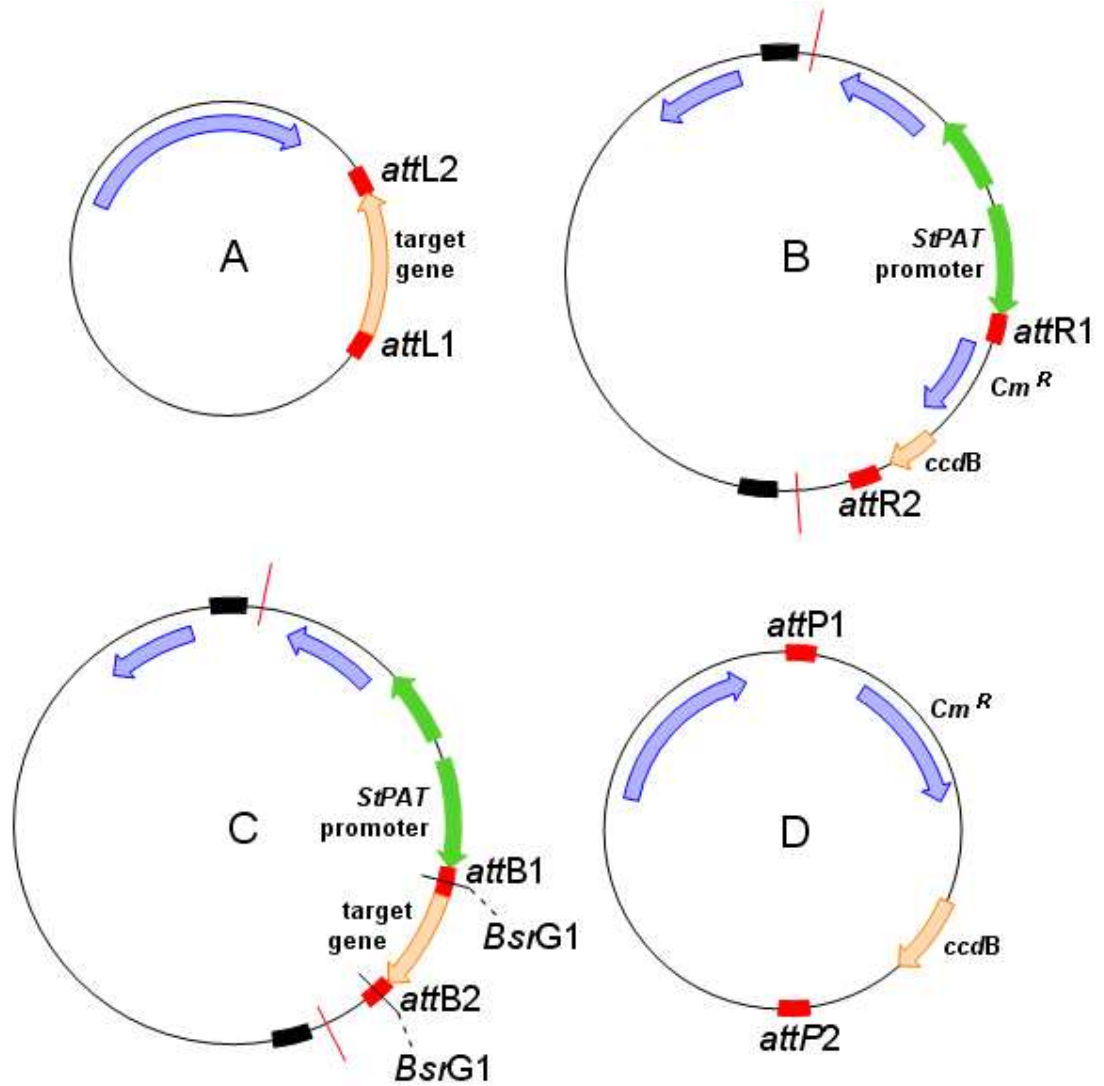


Figure 3.7: The LR reaction – recombination of a pENTR™ plasmid with pCAM PAT GW. Complimentary *att* sites on a pENTR™ plasmid carrying a target gene (A) and pCAM PAT GW (B) permit recombination. This results in an expression plasmid (C) and an unwanted by-product (D).

3.2.7 Construction of reporter expression plasmid and promoter deletions

A reporter expression plasmid was produced to visualise the pattern of expression driven by the *StPAT* promoter *in planta* and was created in the same way as the target gene expression plasmids. The *GusPlus* reporter gene was amplified from pCAMBIA 1305.1 (primer pair 10), adding a portion of the *attB* sites. A second PCR added the full *attB* sites (primer pair 9), and *GusPlus* was then recombined into pDONR™/Zeo in the BP reaction. Colonies containing recombined plasmids were identified through restriction digest with *BsrGI* and after checking the sequence of *GusPlus* in the new pENTR™ plasmid (M13F-20 and M13R universal primers), the LR reaction was carried out with pCAM PAT GW. A *BsrGI* restriction digest again identified colonies containing successfully recombined plasmids, which were subsequently sequenced over the recombination boundaries (DESTSeqF1 and DESTSeqR1 primers) to confirm the successful integration of *GusPlus* into pCAM PAT GW. The reporter expression plasmid was referred to as pDEST™ *GusP*.

StPAT promoter deletion plasmids were constructed to establish which regions of the promoter sequence were necessary to confer activity. They were created by modifying the *GusPlus* reporter expression plasmid, pDEST™ *GusP* (Figure 3.8). The full length *StPAT* promoter was removed by performing a restriction digest with *PstI* and *NcoI* (Figure 3.8A and 3.8B). Three *StPAT* promoter fragments were amplified from pUC19 (deletion 1 – primer pair 11; deletion 2 – primer pair 12; deletion 3 – primer pair 13), adding *PstI* (5') and *NcoI* (3') linkers (Figure 3.8C). These fragments were digested with *PstI* and *NcoI*, purified and then ligated into the digested pDEST™ *GusP* plasmid (Figure 3.8D). 1 µl of ligation mix was used to transform *E. coli* One Shot® TOP10 cells. Cells were cultured on LB agar media with 50 µg/ml kanamycin and resulting colonies were used to inoculate 5 ml liquid LB media with 50 µg/ml kanamycin. Plasmid DNA was extracted from the cells and sequenced (CAMBSeqF1 and CAMBSeqR1) to ensure the *StPAT* promoter fragment did not contain errors and that ligation had occurred as expected. One further promoter deletion was created, where the *StPAT* promoter was completely removed by ligating the overhangs on the *PstI*- and *NcoI*-digested pDEST™ *GusP* plasmid (Figure 3.8B). Although the overhangs were not complementary, the absence of insert in the reaction meant ligation of the *PstI* and *NcoI* overhangs was the only outcome. 1 µl of ligation mix was used to transform *E. coli* One Shot® TOP10 cells. Cells were

cultured on LB agar media with 50 µg/ml kanamycin and colonies were used to inoculate liquid LB media with 50 µg/ml kanamycin. Plasmid DNA was extracted and sequenced (CAMBSeqF1 and CAMBSeqR1) to ensure that ligation had occurred as expected.

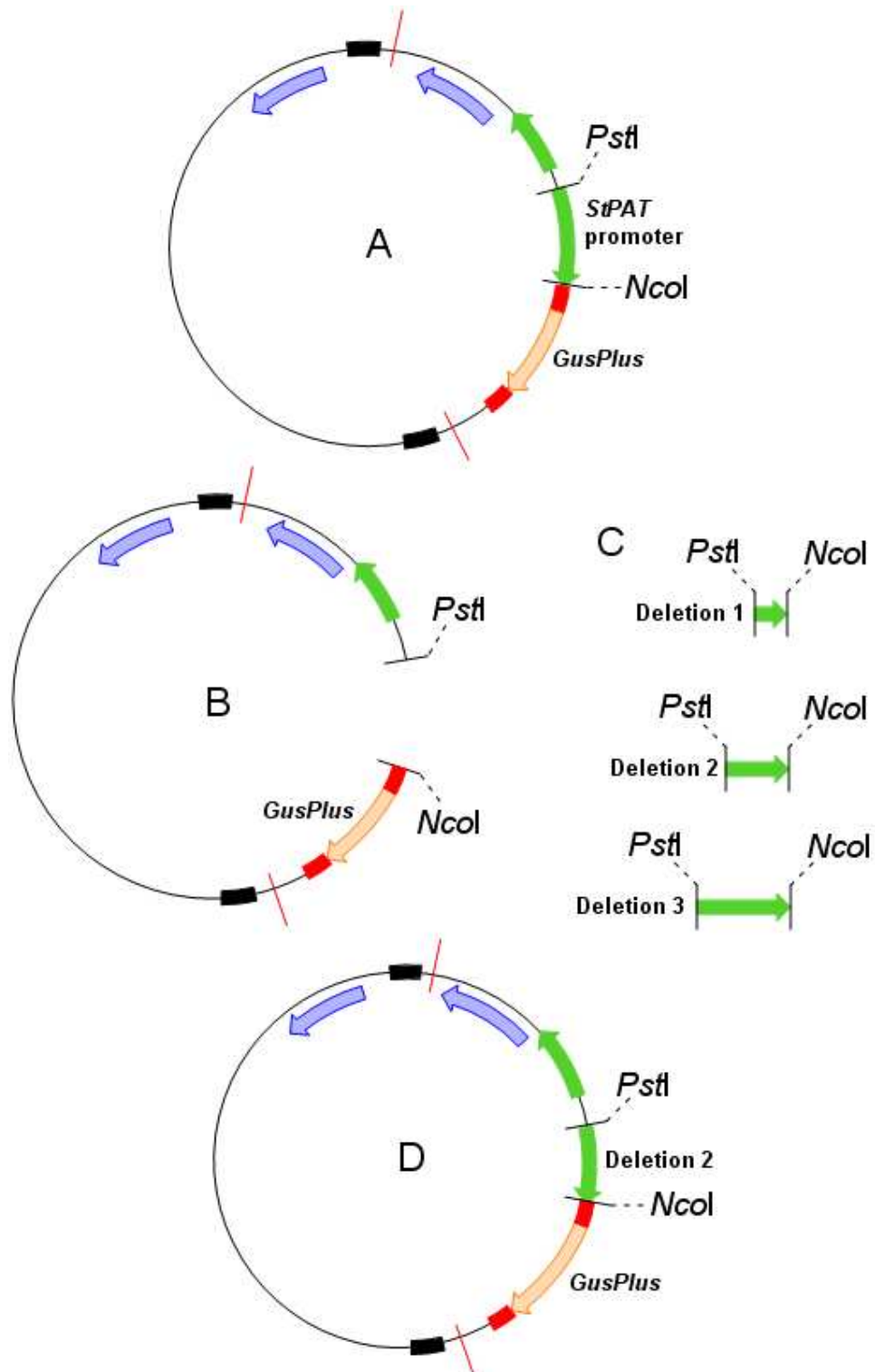


Figure 3.8: The creation of *StPAT* promoter deletion expression plasmids. *pDEST™ GusP* (A) was digested with *PstI* and *NcoI* to remove the full-length *StPAT* promoter. The resulting linearised plasmid (B) was ligated with smaller fragments of the *StPAT* promoter (C) to create promoter deletion expression plasmids (D).

3.3 Discussion

An expression plasmid (pCAM PAT GW) containing the *StPAT* promoter in front of the Gateway[®] cassette was created. This permitted the straightforward and rapid cloning of three target genes and a reporter gene into the expression plasmid using the Gateway[®] system. Should any further target genes be identified, they may be rapidly cloned into the same expression plasmid using the same simple protocols. The reporter expression plasmid, harbouring *GusPlus* under the control of the *StPAT* promoter, was used to create *StPAT* promoter deletion expression plasmids using restriction cloning. *GusPlus* is a synthetic gene based on the sequence of *Staphylococcus* sp. β -glucuronidase and the GusPlus protein is detectable at up to ten-fold higher sensitivity than *E. coli gusA* (Broothaerts *et al.*, 2005). Additionally, the *GusPlus* sequence has been codon optimised for expression in plants (CAMBIA, 2009).

Gateway[®] technology is still a relatively new approach to gene cloning. To date, negative consequences of the technology have not been reported, indicating that researchers using it have not observed unexpected expression levels or patterns. The outcome of introducing the reporter gene and *StPAT* promoter deletion expression plasmids into *Arabidopsis* and tobacco is described in Chapter 4. The result of introducing the target gene expression plasmids into *Arabidopsis* is described in Chapter 5.

4 EXPRESSION ANALYSES OF A PATATIN PROMOTER

4.1 Introduction

4.1.1 Choosing a suitable promoter

The central aim of this investigation is to assess the impact of antioxidant enzymes on alleviating oxidative stress in plant root systems, most notably in reference to post-harvest physiological deterioration (PPD) in cassava. In order to achieve this, a system was designed whereby the genes encoding the antioxidant enzymes were expressed in the roots of the model plant *Arabidopsis thaliana*. To drive root-specific expression in such a system, a suitable promoter was chosen that fulfilled the following criteria. Firstly, the promoter should already have been isolated and sequenced to enable rapid cloning and subsequent expression studies. Secondly, it should direct expression either exclusively or predominantly in storage root tissue in cassava and in root tissue in *A. thaliana* to enable studies in the model system. Finally, it should have been demonstrated that the promoter has successfully been used to drive transgene expression in plant root or tuber tissue.

The promoter of a class I *PATATIN* gene, isolated from potato (*Solanum tuberosum*), met these criteria and so was chosen for this study. The sequences of several different class I *PATATIN* promoters are available on public databases and the promoters themselves are available in the public domain. Storage-root specificity in cassava (Ihemere *et al.*, 2006) and root-specificity in *A. thaliana* (Martin *et al.*, 1997) have been previously documented and class I *PATATIN* promoters are currently widely used by the cassava research community. The promoter of the potato class I patatin gene *B33* (*StB33* promoter) was successfully used to increase pyrophosphatase activity in transgenic potato tubers and thereby accelerate sprouting (Farré *et al.*, 2001). A 1kb promoter version was successfully used to increase ADP-glucose pyrophosphorylase (AGPase) activity in cassava tubers, thereby increasing tuber biomass (Ihemere *et al.*, 2006). This demonstrates proof of concept for this promoter in driving root- or tuber-specific transgene expression.

4.1.2 *PATATIN* gene expression

PATATIN is the generic name given to a family of 40 kD glycoproteins originally isolated from potato (*Solanum tuberosum*). *PATATIN* is the most abundant protein in potato tubers and the genes encoding these proteins are divided into class I and class II depending on the presence (class II) or absence (class I) of a 22 bp insertion in the 5' untranslated region. *PATATIN* gene expression is up-regulated during tuberisation via H4 acetylation (a hallmark of transcriptionally active chromatin) and also by sucrose (Bevan *et al.*, 1986; Stupar *et al.*, 2006). It is most likely that the modification of *PATATIN* gene expression by sucrose occurs via the sucrose-responsive transcription factor Storekeeper that binds a 10 bp motif known as the B-box in the promoter region of *PATATIN* genes (Zourelidou *et al.*, 2002).

An 'A + B repeat' region, spanning approximately 90-100 bp near the 3' end of the promoter, has been inferred in directing tuber-specific and sucrose-responsive *PATATIN* expression (Grierson *et al.*, 1994). In *S. tuberosum*, class I *PATATIN* genes are expressed in all parts of the plant although the level is considerably higher in tubers than in other tissues. The promoters of class II *PATATIN* genes direct expression more evenly throughout all plant tissues. In transgenic *S. tuberosum* plants harbouring the PS20 class I *PATATIN* promoter fused to GUS, the level of GUS activity in tubers was 360-fold that seen in stems, nearly 5,000-fold that in roots and over 500-fold that in leaves (Wenzler *et al.*, 1989). In *A. thaliana*, a species lacking tubers, the *StB33* class I *PATATIN* promoter conferred highly root-specific GUS activity. Activity was also seen at the hydathode at the tip of leaf blades, while expression can be induced in other leaf hydathodes by limiting the plant's nitrogen supply (Martin *et al.*, 1997).

4.1.3 Potential roles of *PATATIN* in planta

Plant tuber storage proteins act as sinks for nitrogen and other essential elements during periods of excess nutrient supply, which subsequently help in the maintenance of the plant during periods of limited nutrient supply. Commonly, tuber storage proteins have an enzymatic activity, allowing them to play other roles in tubers (Shewry, 2003). It is advantageous for tuberous plants if the tuber storage proteins have other roles since only one protein is translated but carries out multiple functions.

PATATIN is known to possess lipolytic acyl hydrolase (LAH) activity (Galliard, 1971; Andrews *et al.*, 1988). The LAH activity of potato tubers is responsible for the rapid hydrolysis of monoacylglycerols (MAG), phospholipids and galactolipids – this activity is actually equivalent to a range of enzyme activities including phospholipase (A1, A2 and B), esterase and glycolipase (Strickland *et al.*, 1995). LAH activity could potentially lead to the production of toxic fatty acids and plant defence compounds such as phytoalexins (via arachidonic acid) or jasmonic acid (via linolenic acid) (Andrews *et al.*, 1988). As a result, a role in defence against biotic and abiotic stress has been inferred for PATATIN. The sequestration of the LAH activity in vacuoles and lysosomes also supports a role during wound responses since the enzyme can only function when released from these organelles upon mechanical wounding (Andrews *et al.*, 1988; Strickland *et al.*, 1995).

4.1.4 Research aims

This chapter is concerned with verifying and fully characterising the expression pattern directed by the 1kb class I *PATATIN* promoter (Ihemere *et al.*, 2006) in *A. thaliana* plants. The promoter, designated here as the *StPAT* promoter, was fused to a reporter gene, *GusPlus* (*GusP*), to enable visual assessment of the pattern of expression. *GusP* activity was monitored in transgenic reporter lines under normal physiological conditions and compared to that previously established (Martin *et al.*, 1997). Since *A. thaliana* does not possess tubers and since class I *PATATIN* promoters confer tuber- rather than root-specificity, it was necessary to confirm whether the particular version of class I *PATATIN* promoter used here exhibited root-specificity activity in *A. thaliana*. Reporter lines were also used to check that the level of expression was sufficient to markedly increase target gene transcript levels. *GusP* activity was then examined under conditions mimicking environmental stresses since a role in stress defence has previously been inferred for PATATIN (Andrews *et al.*, 1988). A novel activity of the *StPAT* class I *PATATIN* promoter was observed and investigated further.

4.2 Results

4.2.1 Generating transgenic *A. thaliana* reporter lines for analysing the expression pattern of a class I *PATATIN* promoter

Before analysing transgenic *A. thaliana* plants over-expressing the three antioxidant target genes (APX, CAT and SOD), it was important to verify that the *StPAT* promoter was directing root-specific expression in *A. thaliana*. The *StPAT* promoter was sequenced in pUC19 prior to cloning, with high quality sequence data retrieved for both strands. The *StPAT* promoter was missing approximately 45 bp at the 3' end (i.e. nearest the native transcriptional start site) compared to the *StB33* promoter (see Appendix, Figure 9.1). It is unknown whether this was due to the original cloning procedure or the *StPAT* promoter simply did not contain this sequence. The missing 45 bp contained the predicted TATA box, although other TATA box sequences were present at -24 and -6 bps relative to the 3' terminus of the *StPAT* promoter. Also, this region is commonly used to classify *PATATIN* promoters as either class I or class II due to the respective absence or presence of a 22 bp insertion (Pikaard *et al.*, 1987). Since this portion of sequence was missing in the promoter used here it was not possible to classify it with certainty as a class I *PATATIN* promoter. However, the successful use of this sequence by others in root-specific over-expression experiments indicated that it was a class I promoter. Furthermore, the sequence data suggested *StPAT* was a class I promoter due to high sequence similarity between this and other class I promoters, particularly at the 3' end (see Appendix, Figure 9.1). The presence of the tuber-specific, sucrose-responsive 'A + B repeat' element and two B-box motifs was demonstrated.

An expression vector containing the *GusPlus* (*GusP*) reporter gene in front of the *StPAT* promoter (Figure 4.1) was used to transform *A. thaliana* Col-0 via the *Agrobacterium*-mediated floral dip method (Clough & Bent, 1998).

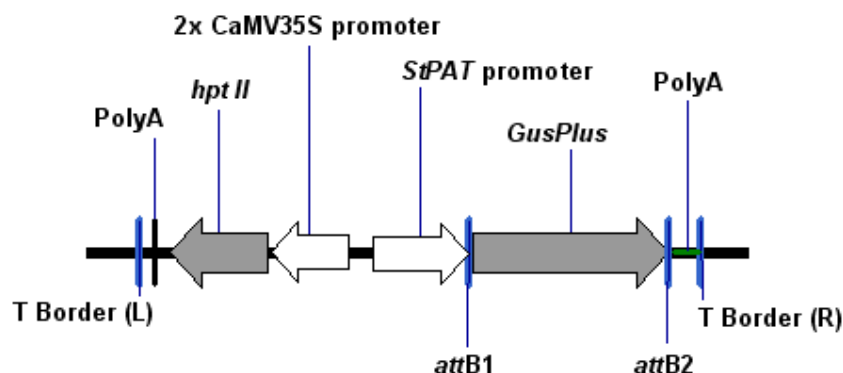


Figure 4.1: T-DNA introduced into *A. thaliana* to generate transgenic reporter lines. The *hptII* gene encodes hygromycin phosphotransferase and confers resistance to hygromycin B.

Screening for positive transformants in the first generation after transformation (T_1) yielded seventeen transgenic lines. All seventeen plants were confirmed as positive via genomic DNA (gDNA) PCR genotyping (primer pair 14). Seven of those seventeen lines were identified as having a single insertion (T_2 generation) and subsequently five of those seven single-insertion lines were shown to be homozygous (T_3 generation). These lines were referred to as *StPAT::GusP* 2-8, 7-1, 10-6, 13-4 and 16-5. Plants grown from T_3 seed were used in assaying for GusP activity. Line *StPAT::GusP* 2-8 was generated first and so was used for initial studies.

4.2.2 The *StPAT* promoter is largely root-specific in *A. thaliana*

The expression pattern of selected *PATATIN* gene promoters has previously been established (Wenzler *et al.*, 1989; Liu *et al.*, 1990). However, the expression pattern of the *StPAT* promoter has not been fully explored and documented. This is essential and will determine if it is suitable for the production of transgenic plants over-expressing antioxidant genes in a root-specific manner. To assess the expression pattern of the *StPAT* promoter in *A. thaliana*, *StPAT::GusP* 2-8 plants were grown *in vitro* on MS media plates under non-stress growth conditions. When assaying whole seedlings or plants, it was evident that GusP activity was chiefly root-specific in the reporter line *StPAT::GusP* 2-8. Intense blue staining of root tissue was coupled with an apparent absence of staining in other tissues (Figure 4.2a). However, closer analysis of stained *StPAT::GusP* 2-8 plants

revealed GusP activity in anthers (Figure 4.2b) and hydathodes, located at the tips of leaf blades (Figure 4.2c). The activity of GusP in all *StPAT::GusP* lines was consistently restricted to these locations.

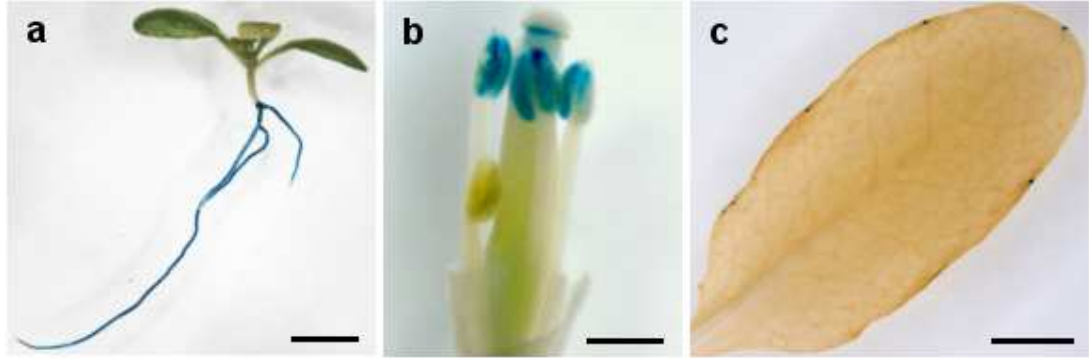


Figure 4.2: The staining pattern of GusP in *StPAT::GusP* 2-8 plants. (a) Whole plant 8 DAG, (b) flower at stage 13 (according to Smyth *et al*, 1990) with all petals and sepals removed, (c) leaf of a mature plant showing GusP staining of the hydathodes. Bars represent 5 mm (a), 0.5 mm (b) and 2 mm (c).

The root-specificity of the *StPAT* promoter closely resembled previous analysis using the *StB33* class I *PATATIN* promoter in *A. thaliana* (Martin *et al.*, 1997). However, the GUS activity seen in anthers in this study has not previously been described for any *PATATIN* promoter. Furthermore, the expression driven by the *StPAT* promoter conferred GusP activity in five hydathodes rather than one. This resembled the expression pattern of a class II *PATATIN* promoter or a class I *PATATIN* promoter acting in a nitrogen-limited system (Martin *et al.*, 1997). It may be that the *StPAT* promoter demonstrates characteristics of both class I and class II *PATATIN* promoters. Importantly though, the *StPAT* promoter used here was chiefly expressed in the root tissue and appeared to be expressed at a high level in this tissue.

4.2.3 *StPAT* promoter-derived GusP activity is activated upon germination and remains stable for eighteen days afterwards

It is important to elucidate the temporal expression pattern of the *StPAT* promoter in order to understand any modifications that may occur in transgenic lines over-expressing antioxidant genes. Furthermore, it is essential for the promoter to be stable throughout a plant's life cycle in order to help it successfully overcome oxidative stress when driving a gene encoding an antioxidant enzyme, since oxidative stress may occur at any time in the field. Localisation of the GusP activity in maturing seeds of *StPAT::GusP* 2-8 plants demonstrated that the promoter was not active before germination (Figure 4.3a – 4.3c), even though the embryonic root apical meristem is initiated at the globular stage (3-4 DAF). However, the differentiation of the three main vascular cell types (phloem, xylem and procambium) is stimulated by germination (Jenik *et al.*, 2007). This differentiation appears to be necessary for the activation of the *StPAT* promoter as GusP activity was visible in root tissue as soon as the radicle emerged from the seed (Figure 4.3d). Root-specific GusP activity was maintained as *StPAT::GusP* 2-8 plants developed (Figure 4.3e – 4.3i).

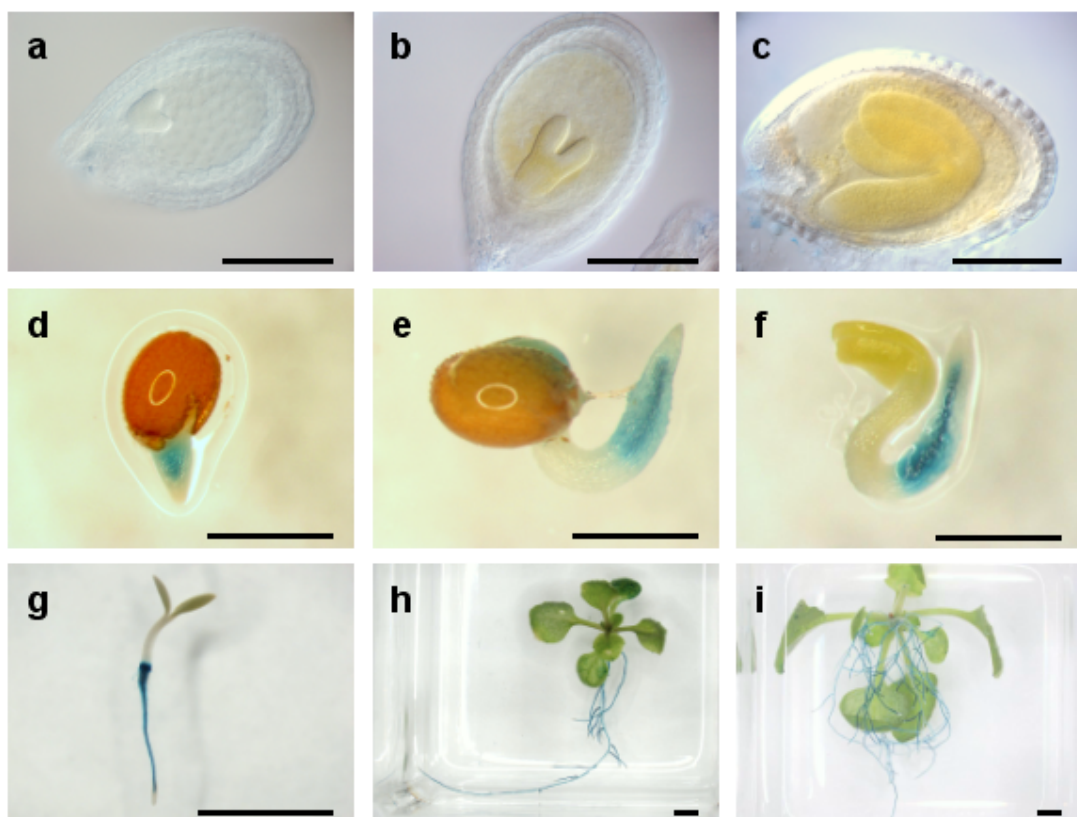


Figure 4.3: Root-specific GusP activity in *StPAT::GusP* 2-8 plants is activated upon germination and maintained until at least 18 DAG. Developing seeds at (a) 5 DAF (heart stage), (b) 6 DAF (early torpedo stage), (c) 8 DAF (up-turned U stage); seedlings at (d) 0.5 DAG, (e) 1.5 DAG, (f) 2 DAG, (g) 3 DAG; and plants at (h) 13 DAG, (i) 18 DAG. Bars represent 0.2 mm (a – c), 0.5 mm (d – f) and 2 mm (g – i).

4.2.4 The *StPAT* promoter is activated during reproductive organ maturation

Since *PATATIN*-derived expression has not previously been established in anthers, it is important to investigate the nature of this expression more fully. This will help enable any phenotypic modifications seen in transgenic plants to be understood, especially regarding fertility. Close examination of Figure 4.2b showed that positive GusP staining was not present in all anthers. *A. thaliana* flowers possess six stamens but only anthers on the four long stamens expressed *GusP* while the two short stamens showed an absence of staining. To analyse the activity of GusP in anthers, flowers of varying ages from soil-grown *StPAT::GusP* 2-8 plants were assayed. In flowers where none of the stamens had elongated,

GusP activity was not seen (Figure 4.4a). Staining was observed in anthers of the four long stamens when the filaments of these stamens had begun to elongate (Figure 4.4b), while staining in anthers of the two short stamens was noted only when their filaments had fully extended (Figure 4.4c and 4.4d). Some GusP activity was present at the base of the stigma (Figure 4.4d) suggesting the *StPAT* promoter is active during the maturation of both male and female reproductive organs.

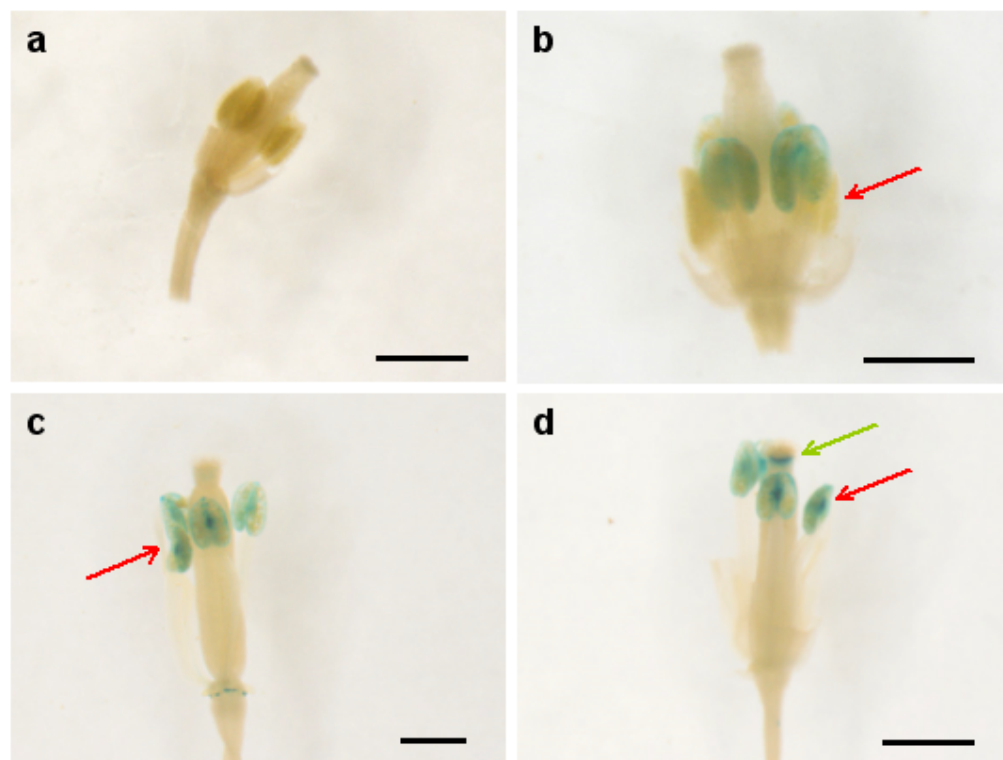


Figure 4.4: GusP activity is activated during the maturation of reproductive organs in flowers of *StPAT::GusP* 2-8 plants. Developmental stages of flowers are according to Smyth *et al.*, 1990: (a) stage 10, (b) stage 10 - 11, (c) stage 12, (d) stage 13. Red arrows indicate anthers of short stamens; a green arrow indicates the base of the stigma. All petals and sepals were removed after GusP assays but before photographing. Bars represent 0.5 mm.

4.2.5 The *StPAT* promoter is wound-inducible

Whilst analysing the activity of GusP in *StPAT::GusP* 2-8 plants, some leaves showed staining that was not restricted to the hydathodes. Furthermore, it was noticed that this staining was most common at sites where leaves had been handled during the assay procedure. This apparent modulation of the expression pattern of the *StPAT* promoter by mechanical wounding was unexpected and must be confirmed. *StPAT::GusP* 2-8 plants were grown *in vitro* on MS media plates under non-stress conditions. Several parts of young plants were artificially wounded and then assayed for GusP activity.

Leaves were pin-pricked four times with the point of sterile tweezers before assaying for GusP. Strong GusP activity was seen where the leaves had been pin-pricked and staining was restricted to a band approximately 0.3 mm wide around the hole left by the tweezers. This indicated that wound-inducible GusP activity was a local rather than systemic event (Figure 4.5a). Flower buds were then squeezed between the fingertips and assayed for GusP. While staining had not been evident in unwounded flower buds, GusP activity was observed in sepals, petals and stems of wounded buds (Figure 4.5b). Trichomes were unusually conspicuous after wounding as a result of positive GusP staining (Figure 4.5c) while developing seeds that had been removed from siliques and so severed from their funiculi also showed intense GusP staining around the wound site (Figure 4.5d). All parts of *StPAT::GusP* 2-8 plants tested resulted in the same intense, local GusP staining around wound sites.

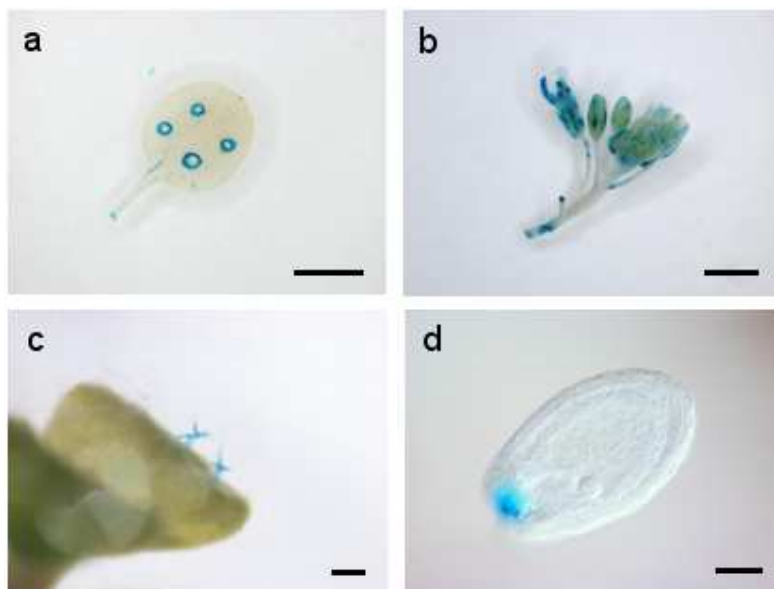


Figure 4.5: Wound-inducibility of the *StPAT* promoter is consistently achieved in different plant parts. (a) A leaf subjected to pin-pricking, (b) an inflorescence subjected to gentle squeezing, (c) wounded trichomes, (d) a developing seed that has been severed from its funiculus (3 DAF, globular stage). Bars represent 3 mm (a – b), 0.5 mm (c) and 0.2 mm (d). Reporter line shown is *StPAT::GusP* 2-8.

It appears that the *StPAT* promoter is conferring localised wound-inducible *GusP* expression in *A. thaliana* reporter lines, which has not previously been noted for any *PATATIN* promoter. However, to validate the novel promoter activity, it must be observed in independent transgenic reporter lines since the observation in only one line may simply be an artefact caused by insertion of the T-DNA into a wound-responsive location in the plant genome.

The five transgenic reporter lines, the result of five independent transgenic events, were grown *in vitro* on MS media plates alongside wild-type and *StB33::GUS* reporter plants. While there was no positive staining in leaves of wild type and *StB33::GUS* plants, the leaves of all five independent *StPAT::GusP* transgenic lines showed wound-localised staining (Figure 4.6). The five lines had varying stain intensities yet all retained the wound-inducible phenotype.

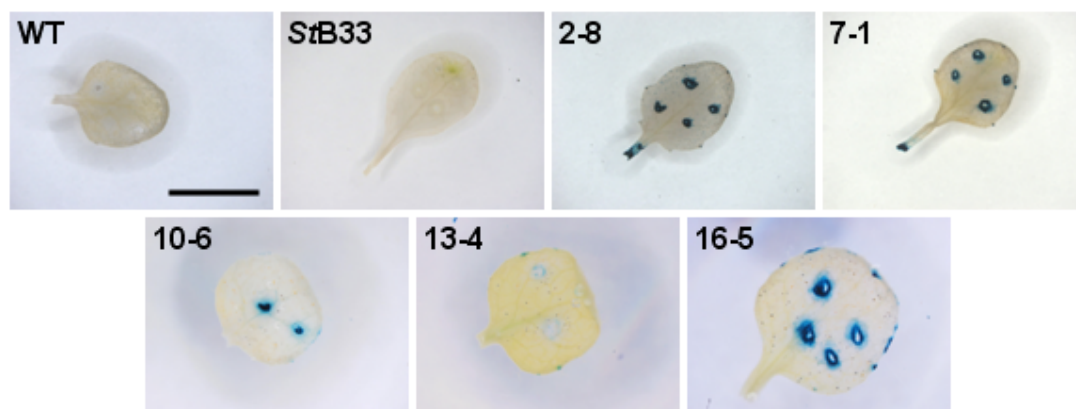


Figure 4.6: Wound-inducibility of the *StPAT* promoter is consistent in independent transgenic *StPAT::GusP* lines. GusP activity in the leaves of wild-type Col-0 plants (WT), *StB33::GUS* plants (*StB33*) and five independent *StPAT::GusP* reporter lines (2-8, 7-1, 10-6, 13-4 and 16-5). Photographs taken at the same magnification, bar represents 10 mm.

Firstly, as the *StB33::GUS* line did not demonstrate wound-inducible GUS activity and the *StPAT::GusP* lines did, we can assume that the *StPAT* promoter sequence contains unique motifs that coordinate this response. Secondly, since the five independent *PAT::GusP* lines all showed wound-inducible GusP activity, it is extremely unlikely that the response is an artefact of the transformation procedure. Furthermore, since the involvement of PATATIN in wound and defence responses has been inferred previously as a result of PATATIN's enzyme activity, it is reasonable to assume this is a real response that merits further investigation.

4.2.6 The *StPAT* promoter is activated rapidly after wounding

By investigating the speed at which GusP activity is induced by a wounding event it may be possible to establish the likely signalling mechanism that brings about the response. To assess this, eight *StPAT::GusP* 2-8 leaves were severely wounded with sterile tweezers and then immersed in GusP staining buffer. One leaf was then immediately rinsed with SDW (0 mins) while the other seven were incubated in the GusP staining buffer for varying periods (as indicated in Figure 4.7) before being rinsed with SDW. Thus the staining seen for each leaf was a

result of cumulative GusP staining for the time indicated in each case. As expected, there was no GusP activity at zero minutes after wounding and GusP staining became more intense as the time after wounding increased. GusP staining was first visible in the leaf tissue only 2 minutes after wounding indicating the inducibility of the *StPAT* promoter is a rapid response (Figure 4.7). The speed of the wound response of the *StPAT* promoter suggests it may be involved with early signalling events during mechanical wounding stress.

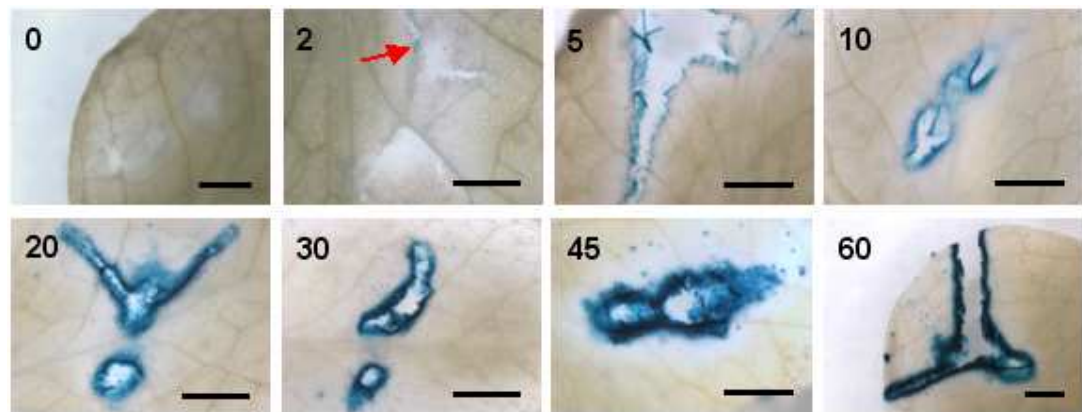


Figure 4.7: Wound-inducible GusP activity in *StPAT::GusP* 2-8 plants over a sixty-minute time-course. Numbers represent time in minutes after wounding. Bars represent 1 mm. Red arrow indicates GusP staining.

4.2.7 The *StPAT* promoter is not activated by low temperature or drought

During different abiotic stresses there is significant overlap between the genes involved in signalling (Mittler *et al.*, 2004), particularly those that are expressed early during stress responses. From the inference that the *StPAT* promoter is activated early during a wound response, it is possible that the promoter may be activated during other abiotic stresses. To address this, the leaves of wild-type, *StB33::GUS* and *StPAT::GusP* 2-8 *Arabidopsis* plants were subjected to four treatments: low temperature, drought, sucrose and wounding. The application of sucrose was performed to verify the sucrose-inducibility of the *StPAT* promoter that is common to all *PATATIN* promoters. A negative control, using *StPAT::GusP* 2-8 material not subjected to the treatments, was included. A wounding treatment was included as a positive control.

Neither low temperature nor drought appeared to cause an increase in GusP activity compared to the negative control (Figure 4.8). The darker colour of

the *StPAT::GusP* 2-8 leaf subjected to drought is due to incomplete clearing rather than GusP activity (all leaves subjected to drought took longer to clear than leaves subjected to other treatments). Sucrose induced a high level of GusP activity in *StPAT::GusP* 2-8 leaves, and did so to a lesser extent in the leaves of *StB33::GUS* plants. This is due to a disparity in reporter gene expression level between the two lines, with the *StPAT::GusP* 2-8 line expressing higher than *StB33::GUS*. The control treatment of mechanical wounding was positive only for *StPAT::GusP* 2-8 as expected.

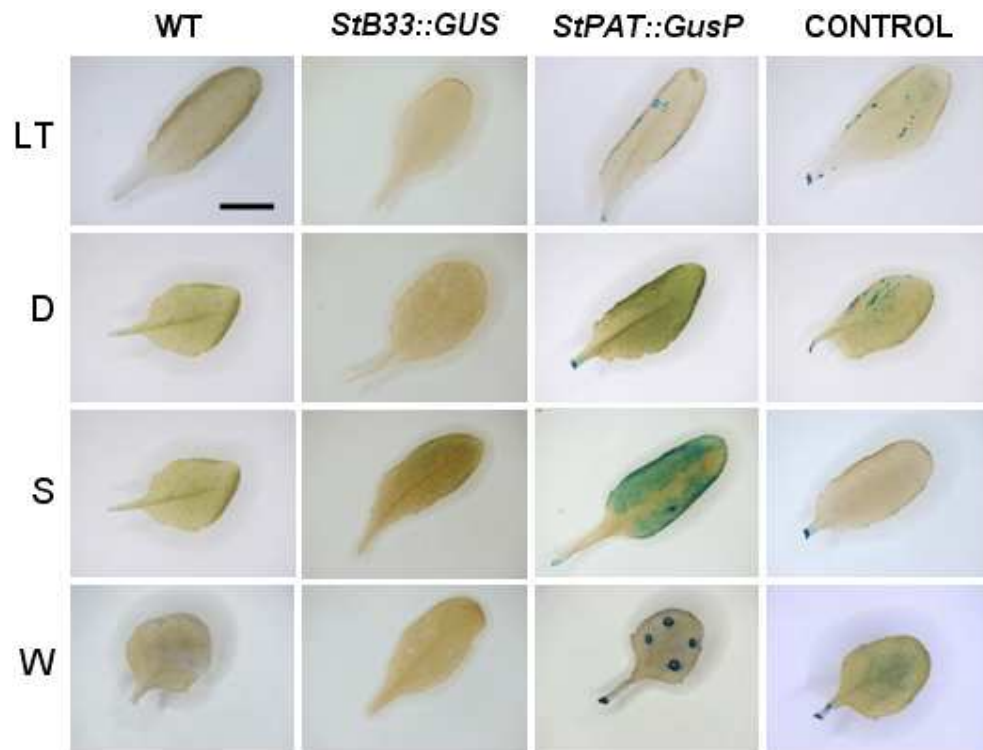


Figure 4.8: Low temperature and drought do not activate two class I *PATATIN* promoters. LT = low temperature (4 °C for 24 hours), D = drought (withheld water for 10 days), S = sucrose (incubated in a 20% sucrose solution for 24 hours), W = wounding (pin-prick or slice immediately prior to GUS assay). WT = wild-type (Col-0), *StPAT::GusP* = *StPAT::GusP* 2-8, control = *StPAT::GusP* 2-8 not subjected to the relevant stress (i.e. negative control). Photographs taken at the same magnification, bar represents 5 mm.

4.2.8 Modulators of wound signalling do not activate the *StPAT* promoter

Of the stress treatments applied in section 4.2.7, only mechanical wounding stimulated GusP activity. To understand why inducibility of the *StPAT* promoter is specific to mechanical stress, chemicals known to be involved in or to stimulate wound signalling were utilised to dissect the response.

Methyl viologen (MV), a bipyridilium herbicide family member, binds to thylakoid and mitochondrial membranes and acts as an electron carrier that ultimately generates the reactive oxygen species (ROS) superoxide (Polidoros *et al.*, 2001). MV and hydrogen peroxide (H₂O₂) were used to simulate an ROS burst, a common early plant response to the majority of abiotic stresses. Silver ions are known inhibitors of ethylene signalling, potentially displacing copper ions in ethylene receptors (Beyer, 1976; Rodríguez *et al.*, 1999). The application of silver thiosulphate here aimed to block the action of ethylene, which has been linked with a role in wound signalling in plants (Saltveit & Dilley, 1978; Boller & Kende, 1980). The addition of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, sought to increase the ethylene levels in the leaf tissues and thereby enhance ethylene signalling. Treatment of leaves with methyl jasmonate (MeJa) simulated the generation of MeJa that accompanies wounding events (Creelman *et al.*, 1992).

No positive GusP staining was seen in the WT control leaves (Figure 4.9). Additionally, the *StB33::GUS* leaves showed an absence of staining. None of the chemical treatments modified the level of GusP staining seen in *StPAT::GusP* 2-8 leaves compared to *StPAT::GusP* 2-8 control leaves, suggesting that the chemicals used were not stimulating the signalling mechanisms underlying the wound-inducibility of the *StPAT* promoter. Any GusP activity observed is a result of accidental damage to the leaves as it is similar in intensity and area between the *StPAT::GusP* 2-8 experimental and control leaves.

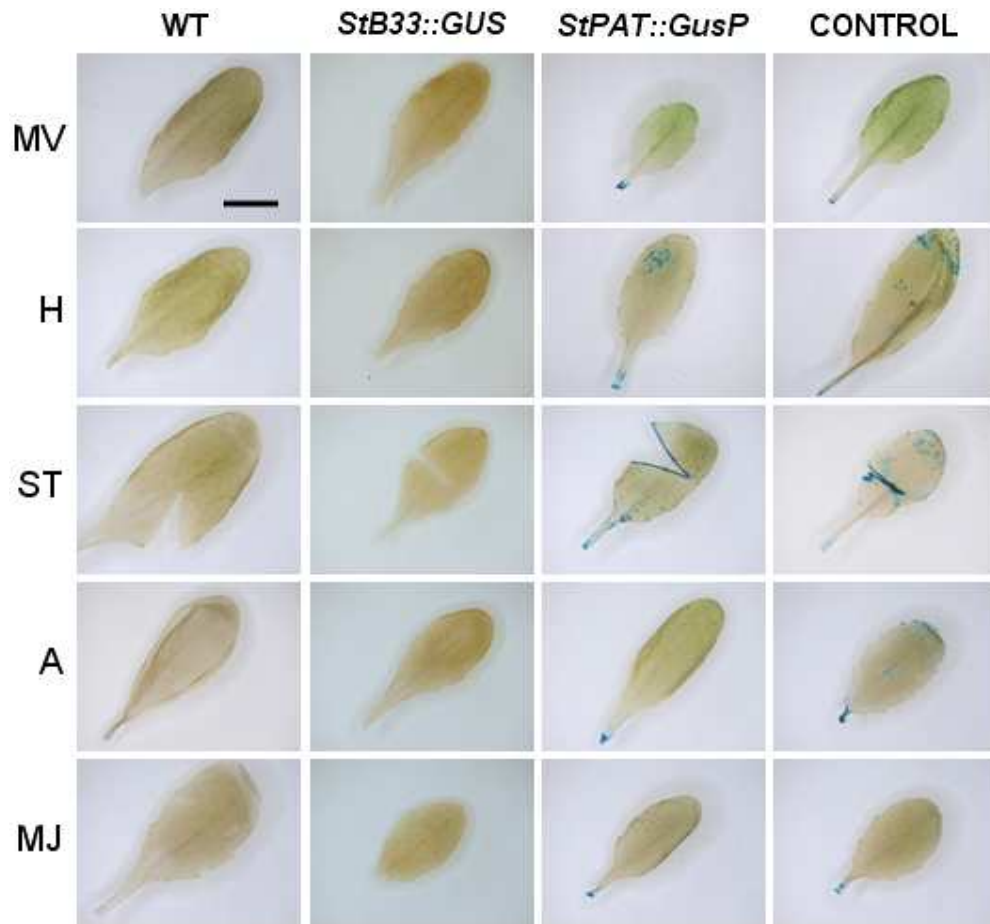


Figure 4.9: Potential wound signalling modulators have no effect on the activity of two class I *PATATIN* promoters. MV = methyl viologen (10 μ M for 1 hour), H = H_2O_2 (100 μ M for 24 hours), ST = silver thiosulphate (20 mM for 1 hour), A = ACC (100 μ M for 24 hours), MJ = methyl jasmonate (100 μ M for 1 hour). WT = wild-type (Col-0), *StPAT::GusP* = *StPAT::GusP* 2-8, control = *StPAT::GusP* 2-8 not subjected to the relevant chemical. Photographs taken at the same magnification, bar represents 5 mm.

Another way of establishing whether the ethylene and jasmonic acid signalling pathways are involved in the wound-inducibility of the *StPAT* promoter is to make use of mutants that are insensitive to ethylene and jasmonic acid.

The recessive jasmonic acid insensitive mutant, *jin1-1*, is the result of a chromosomal rearrangement that leads to the deletion of 30 amino acids at the 3' end of *JIN1* (Roberto Solano, personal communication) (Figure 4.10a). *jin1-1* mutants were crossed with plants of the *StPAT::GusP* 2-8 reporter line. Seed resulting from this cross (*jin1-1/JIN1*, *-GusP*) was grown and allowed to self-pollinate. Seed from the following generation was germinated on a hygromycin-

containing medium to select for lines hemizygous (*-/GusP*) or homozygous (*GusP/GusP*) for the reporter cassette. Positive plants from the hygromycin screening were genotyped via PCR from gDNA (primer pairs 15 and 16, JinF3 with both JinR3 and JinR5, Figure 4.10a). Six plants were identified as homozygous (*jln1-1/jln1-1*) for the jasmonate-insensitive mutation. These six plants were gDNA-genotyped via PCR (primer pair 14) to confirm the presence of the reporter cassette.

The dominant ethylene insensitive mutant, *etr1-1*, harbours a single nucleotide polymorphism (SNP) causing a change in amino acid sequence (Cys65Tyr) of the ethylene receptor gene *ETR1* (Figure 4.10b). The amino acid change prevents the copper cofactor, and consequently ethylene, from binding to the ethylene receptor (*ETR1*) and so confers ethylene insensitivity in a similar manner to the application of silver ions to plant tissue (Bleecker *et al.*, 1988). *etr1-1* mutants were crossed with plants of the *PAT::GusP* 2-8 reporter line. Seed resulting from this cross (*etr1-1/ETR1*, *-/GusP*) was germinated on a hygromycin-containing medium to select for the presence of the reporter cassette. At the rosette stage, gDNA was extracted from 3 plants and a small region at the 5' end of the *ETR1* gene was amplified via PCR (primer pair 17, *ETR1F* and *ETR1R*, Figure 4.10b). The PCR product was subjected to a gel-extraction and the clean product was then sequenced (primer pair 17). High quality sequence data for both strands was obtained for all 3 samples (data for one sample is shown in the Appendix, Figure 9.2). Both an A and a G peak could be seen at the site of the *etr1-1* mutation (base pair 194 of the *ETR1* gene) for the three samples indicating they were all heterozygous for the *etr1-1* mutation (*etr1-1/ETR1*).

The jasmonate- and ethylene-insensitive reporter lines were assayed for *GusP* activity and compared to the *StPAT::GusP* reporter line (Figure 4.10c). None of the six jasmonate-insensitive lines tested showed a reduction in *GusP* staining compared to leaves of control *StPAT::GusP* 2-8 plants (Figure 4.10d). Similarly, there was no reduction in *GusP* staining in ethylene-insensitive reporter lines compared to leaves of control *StPAT::GusP* 2-8 plants (Figure 4.10e).

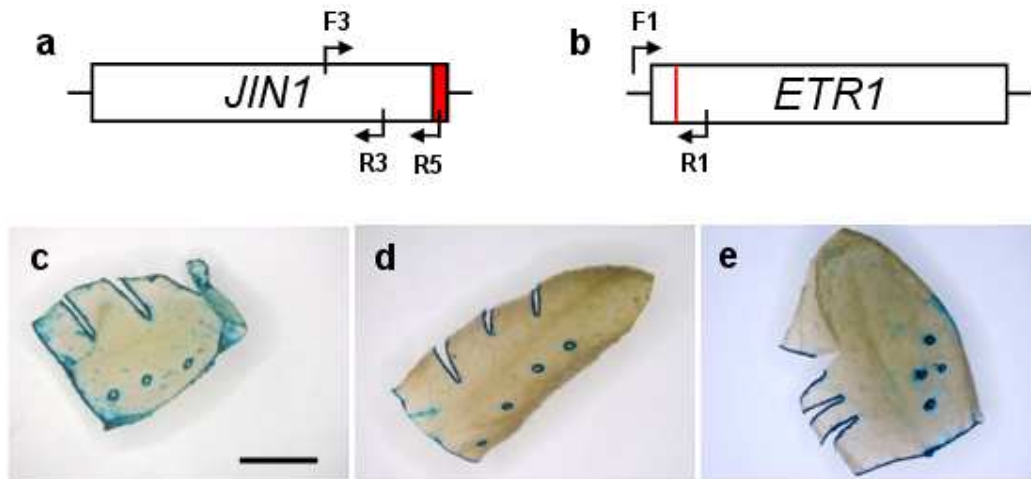


Figure 4.10: Defective phytohormone signalling has no effect on the wound-inducibility of the *StPAT* promoter. Diagrammatic representation of *JIN1* (a) and *ETR1* (b) genes showing respective positions of *jin1-1* and *etr1-1* mutations (red) and primers (arrows). GusP activity in the reporter line *StPAT::GusP* 2-8 (c) and lines defective in jasmonic acid (d) and ethylene (e) signalling. Photographs taken at the same magnification, bar represents 5 mm.

These results confirm the findings from the chemical application assays (Figure 4.9), strengthening the view that either the wound-inducible element of the *StPAT* promoter is not regulated by ethylene or MeJa signalling, or that the promoter is activated upstream of these phytohormones.

4.2.9 Wound-inducibility of the *StPAT* promoter is not a species specific phenomenon

It has been demonstrated that the *StPAT* promoter from potato is wound-inducible in *Arabidopsis*. It is possible however that a promoter introduced from one species into another may behave differently in the new species due to subtle differences in the signals underlying responses to wounding. The possibility of the response we are seeing being species specific must therefore be investigated.

The *StPAT::GusP* reporter cassette was used in the transformation of tobacco (*N. tabacum* SR1) plants. Tobacco was chosen since transformation of this species is relatively simple and seed stocks were available for use. Furthermore, tobacco belongs to the Solanaceae family and so is more closely related to potato than *Arabidopsis*. As a result, studies in tobacco will give a better

indication of the likely behaviour of the *StPAT* promoter in its native species (potato) than the observations made using *Arabidopsis*.

Transformation was *Agrobacterium*-mediated and was performed on 1 cm² leaf discs. Positive transformants were selected for by culturing leaf discs on hygromycin and were subsequently allowed to root and then assayed as whole plants for GusP activity.

All tobacco *StPAT::GusP* lines identified as transformed when screening with hygromycin showed positive staining for GusP activity in root tissue (Figure 4.11a). This activity was restricted only to the root tissue until a wounding treatment was applied (Figure 4.11b). Wound-inducible GusP activity was noted in all tissues wounded by cutting and remained localised in a sharp band approximately 0.3 mm thick around wound sites. Squeezing of leaf tissue (Figure 4.11b) was also sufficient to induce GusP activity.

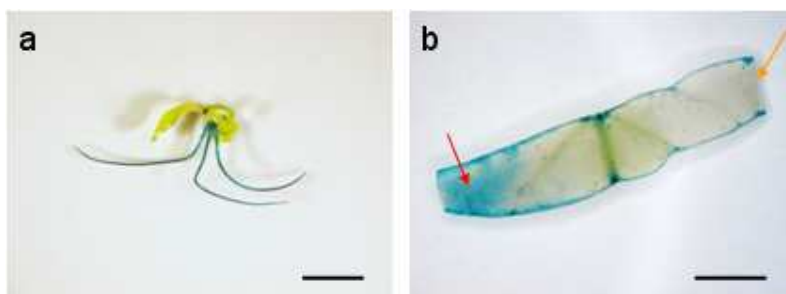


Figure 4.11: The *StPAT* promoter maintains wound-inducibility in *N. tabacum* SR1. GusP activity in transgenic *StPAT::GusP N. tabacum* SR1 plants: (a) root-specific activity, (b) wound-inducible activity in leaf tissue (red arrow – area of squeezing, orange arrow – uncut leaf edge). Bars represent 15 mm (a) and 4 mm (b).

In tobacco, the *StPAT* promoter confers root-specific expression and is activated in non-root tissues by wounding. This result is consistent with the activity of GusP seen previously in *Arabidopsis*, suggesting the wound-inducible activation of the promoter is not a species-specific effect.

4.2.10 The *StPAT* promoter contains an array of *cis* regulatory motifs potentially associated with wound-responsiveness

The wound-inducibility of the *StPAT* promoter is a rapid phenomenon, but as yet the signals responsible for activating this response have not been elucidated. Common transducers of wound signalling, such as H₂O₂, ethylene and MeJa have not reproduced the effect seen on GusP activity after mechanical wounding.

To help establish the signals involved in *StPAT* promoter activation during wounding, a search was carried out within the promoter sequence for *cis* regulatory motifs. The sequence was submitted to the internet-based search tools PlantCARE (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999), with both promoter sequence strands checked for regulatory motifs.

A large number of *cis* elements identified are thought to regulate gene expression in response to environmental cues (Table 4.1). Nine different motifs implicated in responding to light were highlighted through the online database screening, while others implicated in responding to drought, heat and sulphur were also found. Four matches to sugar responsive elements were seen, corroborating the evidence seen here (Figure 4.8) and elsewhere that had previously demonstrated sucrose-inducible *PATATIN* promoter activity. Importantly, fifteen W Box motifs were identified. The W Box sequence binds WRKY transcription factors, which are implicated in responding to many biotic and abiotic stimuli including wounding (Hara *et al.*, 2000; Walley *et al.*, 2007). Also of interest, given the inability of exogenous ethylene and MeJa to induce GusP activity in reporter lines, both ethylene and MeJa responsive *cis* elements were shown to be present within the promoter sequence. Database searching also revealed motifs involved with responses to the phytohormones abscisic acid and gibberlin. The presence of two B box elements, identified previously via sequencing (Section 4.2.1), was confirmed. A large number of additional motifs were identified (see Appendix, Table 9.1) but were ignored here as they were considered irrelevant concerning the wound-inducibility of the *StPAT* promoter.

The *SPORAMIN* promoter from sweet potato (Wang *et al.*, 2002) shares many similarities with the *StPAT* promoter. Both are derived from a tuberous plant species and the product of the *SPORAMIN* and *PATATIN* genes are the most abundant proteins in the tubers of the relative species. Furthermore, the activity of the two promoters is inducible by mechanical wounding and the *SPORAMIN* and *PATATIN* proteins both possess enzyme activity. Given these parallels, it is likely

that similar signals are responsible for the wound-inducible activity of the *StPAT* and *SPORAMIN* promoters. To establish if any *cis* motifs are shared between the promoters, the *SPORAMIN* promoter sequence (both strands) was submitted to PlantCARE (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999). Identified elements were compared by eye to the results for the *StPAT* promoter and matching motifs were highlighted in bold in Table 4.1 (and in the Appendix, Table 9.1).

Four elements associated with responding to light were present in both promoters, implying that light plays a key regulatory role in the physiology of tuberosous tissue. The HSE heat stress responsive element was over-represented in the two promoters with six motifs found in the *StPAT* promoter and three in the *SPORAMIN* promoter. W Box motifs were over-represented too, with 15 and 7 matches respectively. Drought and general stress responsive motifs were also common to both promoters.

Table 4.1: Putative *cis* regulatory motifs within the *StPAT* promoter sequence.

Motif Function	Motif Name	Motif Sequence	M	N
Absciscic acid responsiveness	ABRE	TACGTG	6/6	1
Drought responsiveness	MBS	CAACTG	6/6	2
Ethylene responsiveness	ERE	ATTTCAAA	8/8	1
Gibberellin responsiveness	GARE	AAACAGA	7/7	1
Heat stress responsiveness	CCAAT box	CCAAT	5/5	1
	HSE	AAAAAATTTTC	9/10	6
Light responsiveness	3-AF1 binding site	AAGAGATATTT	10/11	1
	AAAC motif	CAACAAAAACCT	11/12	1
	ACE	AAAACGTTTA	9/10	1
	ATCT motif	AATCTAATCT	9/10	1
	Box I	TTTCAA	7/7	1
	G Box	CACGTC	6/6	1
	I Box	GATAAGGCG	9/9	1
	Sp1	CC(A/G)CCC	5/6	5
	TCCC motif	TCTCCCT	7/7	1
MeJa responsiveness	TGACG motif	TGACG	5/5	5
Storekeeper binding	B Box	GCTAAACAAT	10/10	2
Stress responsiveness (general)	TC-rich repeat	ATTTTCTTCA	9/10	1
	W Box	(T)(T)TGAC(C/T)	n/a	15
Sugar responsiveness	SURE	AATAGAAAA	9/9	2
	SURE2	AATACTAAT	9/9	2
Sulphur responsiveness	SRE	GAGAC	5/5	2

M = best match to motif

N = number of best matches

Bold = also found in *SPORAMIN* promoter sequence

While the PlantCARE and PLACE databases contain a comprehensive array of known *cis* regulatory motifs, there are other important elements that are not included therein. A search of the literature was performed that aimed to identify any wound-related motifs that were not integrated into the online databases.

The *NOPALINE SYNTHASE* gene (*NOS*) is induced upon wounding in a range of tobacco tissues and like the *StPAT* promoter, the *NOS* promoter is active in reproductive organs (An *et al.*, 1990). Furthermore, deletion of the TATA box did not affect the wound-inducibility of the *NOS* promoter (the *StPAT* promoter does not have its native TATA box, but does contain other potential TATA boxes). Promoter deletion analysis identified a 10 bp 'Z element' that appeared to confer wound-inducible activity on the *NOS* promoter (GCACATACGT).

The expression of the potato *PROTEINASE INHIBITOR IIK* (*PI IIK*) gene is regulated by cell wall-derived oligogalacturonides and expression also increases in response to wounding. The effect of a wound event on *PI IIK* expression is systemic rather than local. Deletion analysis and nuclear protein binding assays demonstrated a 10 bp motif (AAGCGTAAGT) was essential for wound-inducibility (Palm *et al.*, 1990).

The promoter of the tobacco *S-LIKE RIBONUCLEASE 1* (*NGR1*) gene contains a 9 bp sequence (TAATTACTC) designated as the WUN motif that has been implicated in wound-related changes in *NGR1* expression (Hayashi *et al.*, 2003). The *NGR1* promoter sequence contains fourteen perfect or near matches to WUN motifs. Deletion of the only perfectly matched WUN motif leads to abolishment of wound-induced *NGR1* gene expression.

The tobacco *PEROXIDASE* (*TpoxN1*) gene is vascular system specific and wound-inducible (Sasaki *et al.*, 2006). Like the *StPAT* promoter, activity of the *TpoxN1* promoter cannot be induced artificially by stimulating MeJa or ethylene signalling pathways. Promoter deletion analysis and nuclear protein binding assays have revealed that a 14 bp motif, termed the vascular system-specific and wound-responsive element (VWRE, sequence GAAAAGAAAATTTC) is sufficient to confer wound-inducibility on the *TpoxN1* promoter and that sequence mutations within this region dramatically reduced the activity. The palindromic sequence AAATTT within the VWRE is essential for nuclear protein binding.

A microarray comparing unwounded *Arabidopsis* leaves with leaves that had been wounded and left for five minutes identified one hundred and sixty-two genes whose expression was up-regulated at least two-fold (Walley *et al.*, 2007). These were termed rapid wound responsive (RWR) genes and their promoter regions were examined for commonly occurring motifs. The 6 bp rapid stress response element (RSRE, sequence CGCGTT) was over-represented in the 5' untranslated regions of RWR genes and the fusion of four RSREs and a minimal promoter to the luciferase reporter gene yielded *Arabidopsis* lines with wound-inducible luciferase activity.

Both strands of the *StPAT* promoter sequence were screened for the presence of the five elements described above using Vector NTI 10. The best matches are shown below in Table 4.2. The *SPORAMIN* promoter was also screened with the same number of mismatches as seen in matches with the *StPAT* promoter and elements common to both promoters are highlighted in bold text. All five elements were present in the *StPAT* promoter with at least 70%

homology to the stated motif sequence. Of these, the PI IIK motif, RSRE and Z element were also found within the *SPORAMIN* promoter, potentially underlining their importance in modulating wound-inducible promoter activity.

Table 4.2: Putative wound-responsive *cis* motifs located in the *StPAT* promoter identified through literature searching.

Motif Name	Reference	Motif Sequence	M	N
PI IIK motif	Palm <i>et al</i> (1990)	AAGCGTAAGT	7/10	7
RSRE	Walley <i>et al</i> (2007)	CGCGTT	5/6	3
VWRE	Sasaki <i>et al</i> (2006)	GAAAAGAAAATTTC	12/14	1
WUN	Hayashi <i>et al</i> (2003)	TAATTACTC	8/9	1
Z element	An <i>et al</i> (1990)	GCACATACGT	7/10	5

M = best match to motif

N = number of best matches

Bold = also found in *SPORAMIN* promoter sequence

In the reporter T-DNA there are forty-two base pairs between the end of the *StPAT* promoter and the translational start site of the *GusP* gene. Within this region lies sequence associated with the process of cloning via the Gateway[®] system, namely an *attB1* site. To determine if this sequence might confer wound-inducibility on the *StPAT* promoter, both strands of the sequence from the start of the promoter up to the translational start point of the *GusP* gene was submitted to the PlantCARE (Lescot *et al.*, 2002) and PLACE (Higo *et al.*, 1999) databases. The sequence was also screened for the motifs identified via literature searching. Six motifs were identified in the Gateway[®]-associated region further to those identified using only the *StPAT* promoter sequence, with one associated with wound-responsiveness (Table 4.3). Five of the six motifs had already been found in the *StPAT* promoter sequence – only the copper responsive element (CuRE) was novel.

Table 4.3: Putative *cis* regulatory motifs in the sequence between the *StPAT* promoter and the *GusP* gene in the reporter T-DNA.

Motif Function	Motif Name	Motif Sequence	M	N
Anaerobic fermentation	Anaero3	TCATCAC	7/7	1
Copper responsiveness	CuRE	GTAC	4/4	1
Protein binding (general)	DOFcore	AAAG	4/4	1
Pollen specificity	GTGA(Nt)	GTGA	4/4	1
Plastid gene expression	S1F box	ATGGTA	6/6	1
Wound responsiveness	Z element	GCACATACGT	7/10	1

M = best match to motif

N = number of best matches

Bold = also found in *SPORAMIN* promoter sequence

4.2.11 Wound-inducible promoters harbour common *cis* regulatory motifs

The *cis* elements described above (Section 4.2.10) that were found in the *StPAT* promoter sequence are known regulatory motifs. However, it is also possible that the wound-inducibility of the *StPAT* promoter is conferred by an uncharacterised *cis* motif. The sequence of the *StPAT* promoter was compared with other wound-inducible promoters to establish whether they shared common motifs. Promoters were chosen for comparison to that of *StPAT* on the basis that they have been shown to be wound-inducible and that their sequences were accessible – these were the promoters of *SPORAMIN* (*lSPN*) (Wang *et al.*, 2002), *BvXTH2* (Dimmer *et al.*, 2004), *StBEL5* (Chatterjee *et al.*, 2007) and *ZmMPI* (Cordero *et al.*, 1994). The sequence comparison was performed by the Weeder web tool (Pavesi *et al.*, 2006). The output from the Weeder analysis consisted of the best ten motifs of six, eight, ten and twelve bases long on both the forward and reverse strands. The five promoter sequences were checked against these forty motifs using Vector NTI 10, with the best matches across all five promoters shown in Table 4.4. The sequences were given the arbitrary name Weeder-derived motif (WDM) and then numbered.

WDM1 was the only motif present as an exact match in all five promoters, suggesting it may play a crucial role in wound responsiveness. Of the seven interesting motifs identified by Weeder, only two matched directly with a region of a known motif in the PLACE or PlantCARE databases. WDM2 matched part of a light responsive motif (L box), while WDM3 matched part of an endosperm specific

motif (Prolamin box). The other sequences highlighted by Weeder could potentially be novel regulatory motifs involved in wound-responsiveness.

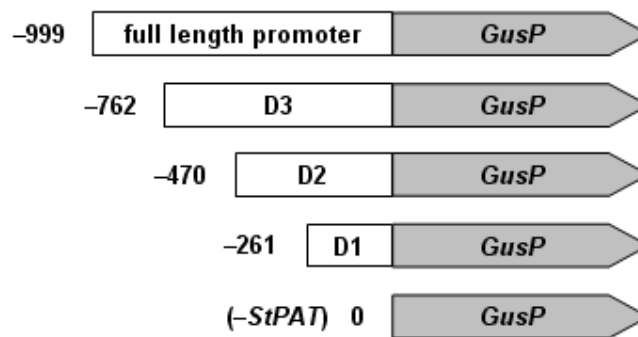
Table 4.4: Conserved *cis* sequences in wound-inducible promoters.

Sequence	Name	Best match to promoter sequence					PlantCARE or PLACE consensus
		<i>StPAT</i>	<i>lbSPN</i>	<i>StBEL5</i>	<i>ZmMPI</i>	<i>BvXTH</i>	
AGGTGC	WDM1	6/6	6/6	6/6	6/6	6/6	
TGGGGT	WDM2	6/6	6/6	6/6	5/6	6/6	<u>TGGGGT</u> TGGTGAGA
GTAGTG	WDM3	6/6	5/6	6/6	6/6	6/6	<u>tgtagTG</u> TAAAGtgaaa
ATACGCAC	WDM4	7/8	7/8	8/8	8/8	7/8	
TATACCATGC	WDM5	10/10	9/10	8/10	9/10	10/10	
TAATGGACAGCA	WDM6	10/12	9/12	12/12	10/12	11/12	
GTGCGTATATAC	WDM7	10/12	10/12	11/12	12/12	9/12	

4.2.12 The 3' 261 bp of the *StPAT* promoter is sufficient for wound-inducibility

A large number of *cis* regulatory motifs potentially involved in wound-responsiveness have been identified in the *StPAT* promoter sequence. To help pinpoint which motifs have an effect on promoter activity *in planta*, *StPAT* promoter deletions were created. In addition to the full length promoter, four *StPAT* promoter fragments of different sizes were generated and ligated in front of the *GusP* gene in the reporter cassette (see Figure 4.12A). These cassettes were then used to transform both tobacco and *Arabidopsis*. gDNA was extracted from transformed plants and used as template for PCR genotyping (primer pair 18, results for tobacco shown in Figure 4.12B, results for *Arabidopsis* not shown).

A



B

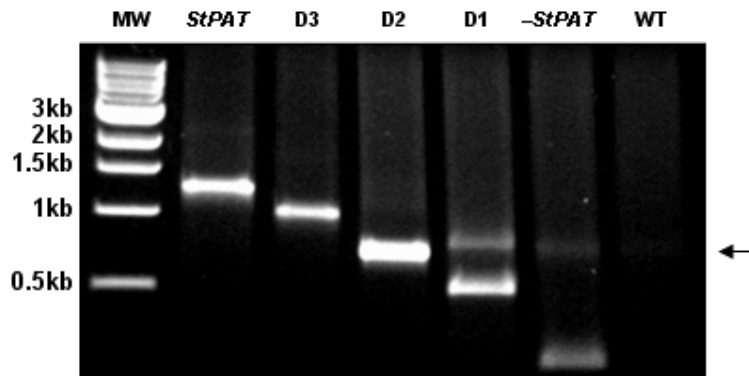


Figure 4.12: *StPAT* promoter deletions. **A:** sizes in base pairs of each promoter deletion. **B:** agarose gel showing results from gDNA genotyping of transgenic tobacco plants. MW = 1kb molecular weight marker (NEB), *StPAT* = full length *StPAT* promoter (amplicon = 1,302bp), D3 = deletion 3 (1,065bp), D2 = deletion 2 (773bp), D1 = deletion 1 (564bp), -*StPAT* = promoter-less cassette (301bp), WT = wild-type SR1 (no product). Arrow denotes PCR product from non-specific primer binding in lanes D1, -*StPAT* and WT.

Lines confirmed as transgenic were tested for *GusP* activity in root tissue. Lines positive for root-specific *GusP* activity were then tested for wound-inducibility of the *StPAT* promoter in leaf tissue (Figure 4.13).

In *Arabidopsis*, wound-inducibility of the *StPAT* promoter was only abolished when the whole promoter sequence was deleted from the reporter cassette (Figure 4.13A). D1 (-261bp) is sufficient to maintain wound-inducibility of *StPAT* indicating the regulatory motifs responsible for conferring this activity are present in the 3' 261bp of the promoter. Importantly the promoter-less reporter cassette was insufficient to cause wound-inducible *GusP* staining. This cassette still contained the forty-two base pairs of Gateway[®]-associated sequence directly

upstream of the translational start site of *GusP*, suggesting this sequence was not involved in the wound responsiveness of the *StPAT* promoter.

The equivalent tobacco deletion lines showed the same staining pattern as their *Arabidopsis* counterparts (Figure 4.13B), with positive staining in *StPAT*, D3, D2 and D1. For both plant species, there was an absence of staining in $-StPAT$ and wild-type lines.

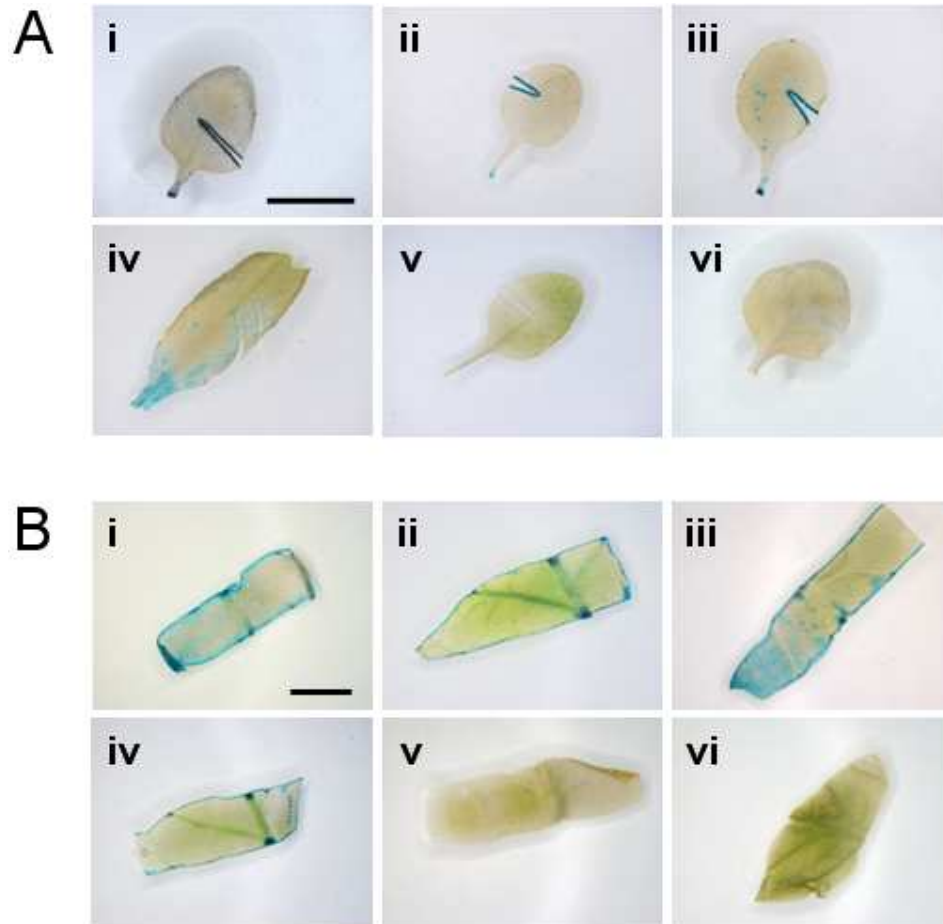


Figure 4.13: A 261bp fragment of the *StPAT* promoter is sufficient to maintain wound-inducible *GusP* activity. (A) *A. thaliana* and (B) *N. tabacum*: (i) full length promoter, (ii) deletion 3, (iii) deletion 2, (iv) deletion 1, (v) promoter-less cassette, (vi) wild-type Col-0 (A) and SR1 (B). Bars represent 5 mm.

4.2.13 The D1 *StPAT* promoter fragment contains wound response-related *cis* regulatory motifs

It is common for the 3' end of a promoter (closest to the gene it controls) to assert the biggest influence on gene expression. In agreement with this, the 3' 261bp of the *StPAT* promoter is sufficient to confer wound-inducible activity. This sequence was checked for the presence of the previously identified wound response-related motifs using Vector NTI 10. Of the original wounding-related motifs shown in Tables 4.1, 4.2 and 4.4, nineteen separate instances were found in the D1 fragment of the promoter (Figure 4.14). Four Weeder-derived motifs (WDM4, WDM5, WDM6 and WDM7) were located within one hundred base pairs of the translational start site of *GusP*. Other wounding-related *cis* elements found within the D1 promoter fragment included a W box, the VWRE, a z-element and two PI I/K motifs.

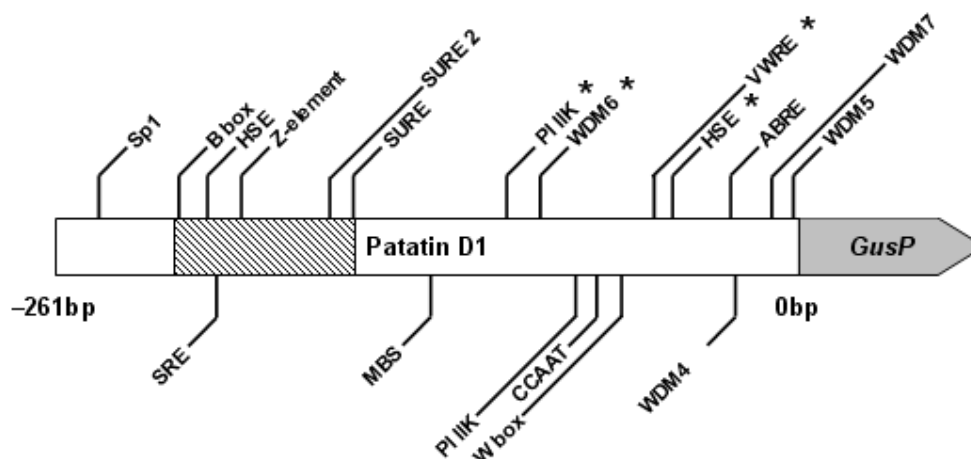


Figure 4.14: The D1 fragment of the *StPAT* promoter contains a large number of wound response-related *cis* regulatory motifs. Position of motifs is approximately to scale. Shaded area is the tuber-specific, sucrose responsive region. Motifs above the promoter are on the forward strand, motifs below the promoter are on the reverse strand. Asterisks denote motifs modified in the *StB33* promoter due to sequence conflicts with the *StPAT* promoter.

The difference in wound-inducibility of the *StB33* and *StPAT* promoters is most likely due to differences in the sequence of the promoters. Since the D1 fragment is sufficient to confer wound-inducible activity on the *StPAT* promoter,

the D1 sequence was aligned with the *StB33* promoter sequence using Vector NTI 10. The two sequences shared 91.7% homology over the D1 region and there were seven points of conflict between the two promoters in the D1 region: five single nucleotide conflicts, one four base pair sequence present in *StB33* but not in *StPAT* and one thirteen base pair sequence in *StPAT* but not *StB33*.

A single nucleotide conflict at –52 bp occurred within the sequence of both the VWRE and an HSE on the forward strand (Figure 4.14). Similarly, another single nucleotide conflict at –89 bp occurred within the motif sequence of the WDM6 motif. Furthermore, the thirteen base pair sequence seen in *StPAT* but not *StB33* closely mirrored the sequence of the PI IIK element on the forward strand. These sequence differences could account for the different responses to wounding between the two promoters.

4.2.14 Changes in GusP activity are not detected at the mRNA level

Rapid changes in GusP activity have been detected in *StPAT::GusP* reporter lines upon wounding in both *Arabidopsis* and tobacco. It is not known however if the increase in GusP activity is due to *de novo* transcription of the *GusP* gene, or if a post-transcriptional control on protein translation or enzyme activation is being relaxed. To investigate this, *Arabidopsis GusP* mRNA levels were assessed in a semi-quantitative manner using PCR (sqPCR). cDNA was synthesised from total RNA extracted from *StPAT::GusP* 2-8, *StB33::GUS* and wild-type plants. Total RNA was extracted from unwounded leaves and leaves that had been severely wounded thirty minutes previously for each line. A thirty minute time period was chosen as strong GusP staining was seen after this time in *StPAT::GusP* 2-8 leaves (Figure 4.7). Since *StPAT::GusP* 2-8 and *StB33::GUS* plants harboured different versions of the β -glucuronidase reporter gene (*GusPlus* and *uidA* respectively), two different amplifications were necessary.

There was a very slight increase in *GusP* mRNA level in the wounded *StPAT::GusP* 2-8 samples, but levels were very similar between unwounded and wounded leaves, unlike the results seen in histochemical staining assays which showed an increase over the same time course in the wounded samples (Figure 4.15). Amplification of *GUS* demonstrated a substantial increase in transcription in wounded *StB33::GUS* leaves, again conflicting with earlier GusP staining results. It should be emphasised there was no opportunity for confusing the *StPAT::GusP* 2-8 and *StB33::GUS* samples due to the difference in the version of reporter gene

used to generate the two lines. No amplicons were observed in the wild-type samples.

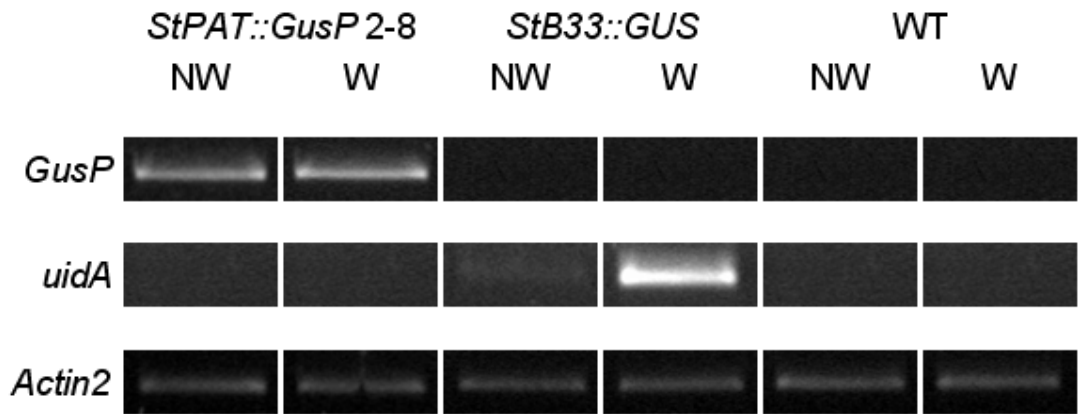


Figure 4.15: A wounding treatment is insufficient to increase *GusP* mRNA levels in *StPAT::GusP* leaf tissue. NW = no wounding, W = 30 minutes after severe wounding. *GusP* = *GusPlus*, *uidA* = *E. coli* β -glucuronidase (NCBI accession: NC_010498). cDNA levels normalised with *Actin2* (At3g18780).

Given the unexpected nature of the results seen above (Figure 4.15), the semi-quantitative RT-PCR was repeated for the *StPAT::GusP* samples. Unwounded leaf samples were compared with samples wounded two hours before RNA extraction. The time period between wounding and RNA extraction was extended to maximise the chance of detecting a change in the expression level of *GusP*. No difference in expression level was observed between unwounded and wounded leaf samples (Figure 4.16).

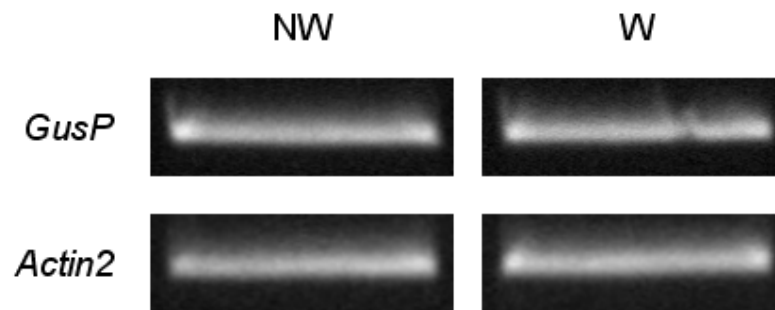


Figure 4.16: Expression level of *GusP* in the leaves of reporter line *StPAT::GUS* 2-8 analysed by sqPCR. NW = no wounding, W = 2 hours after severe wounding. *GusP* = *GusPlus*, cDNA levels normalised with *Actin2* (At3g18780).

4.3 Discussion

The *StPAT* promoter conferred largely root-specific reporter gene expression in *Arabidopsis* and tobacco. Inducibility of the promoter was observed almost immediately after a wounding event, with reporter activity restricted to the wound site. Phytohormones, ROS and other abiotic stresses were unable to induce the *StPAT* promoter. A 261 bp fragment of the promoter was sufficient to confer wound-inducibility, and contained numerous potential wound-responsive *cis* regulatory motifs.

4.3.1 The relationship between PATATIN enzyme activity and function

A role in responding to wound events has been inferred for PATATIN here and elsewhere previously (Andrews *et al.*, 1988). The response seen here can be assumed to be real since it was observed in two plant species and multiple independent reporter lines.

It is common for plant storage proteins to have a secondary role. Most tuberous crop species possess a major tuber storage protein like PATATIN in potato, and the majority of these proteins possess enzyme activity.

The primary storage protein of sweet potato tubers is SPORAMIN, as referred to earlier in this chapter. SPORAMIN exhibits dehydroascorbate reductase and monodehydroascorbate reductase activities (Hou & Lin, 1997), implying a role in oxidative stress defence. SPORAMIN is also a trypsin inhibitor (Hou & Lin, 2002), suggesting a role in stress signalling or senescence-related phenomena. Furthermore, the *SPORAMIN* promoter is wound-inducible (Wang *et al.*, 2002) providing further evidence for a role in defence. In contrast to the *StPAT* promoter, the *SPORAMIN* promoter is induced by exogenous MeJa, suggesting it is activated downstream of this phytohormone *in planta*. DIOSCORIN is the major storage protein of yam tubers and has similar enzymatic properties to SPORAMIN. It too possesses DHA and MDHA activity (Hou *et al.*, 1999a), and can also inhibit trypsin although to a lesser extent than SPORAMIN (Hou *et al.*, 1999b). Protein sequence alignments suggested DIOSCORIN was a member of the carbonic anhydrase family, albeit with active site alterations (Hewett-Emmett & Tashian, 1996). DIOSCORINS isolated from a number of yam species were later proved to have carbonic anhydrase activity (Hou *et al.*, 1999b), which is associated with the maintenance of efficient photosynthesis. The major storage protein of taro, G2 globulin, also has enzyme activity (Shewry, 2003).

PATATIN possesses an enzyme activity and so follows the trend of tuber storage proteins. It is classified as a lipolytic acyl hydrolase (LAH) (Galliard, 1971) and acts to hydrolyse a wide variety of acyl lipids. It has been stated that the activity of PATATIN is equivalent to the activity of a combination of other enzymes such as esterase, phospholipase and glycolipase (Strickland *et al.*, 1995). The significance of the enzyme activity of PATATIN is unproven although possible functions, especially related to biotic stress responses, have been put forward.

Corn root worm (*Diabrotica*) larvae fed with purified PATATIN (500µg/g) added to standard diet showed growth rates eighteen-fold lower than controls (Strickland *et al.*, 1995). This effect was attributed to the enzyme activity of PATATIN and supports a role in defence against biotic stresses. The phospholipase or LAH activity of PATATIN can act on membrane lipids to release linolenic acid (LA), a precursor for jasmonate biosynthesis (Creelman & Mullet, 1995; Scherer, 2002). This indicates PATATIN may act upstream of jasmonate signalling, a theory supported by: the inability of MeJa to stimulate GusP activity under the control of the wound-inducible *StPAT* promoter in reporter lines; jasmonate-insensitive reporter lines that retained wound-inducible GusP expression; the localised activity of GusP rather than the characteristic systemic response mediated by MeJa. The localised nature of GusP expression also indicates a function for PATATIN in healing rather than defence signalling to prevent further wounding (León *et al.*, 2001). The breakdown of membrane lipids is one of the earliest signals during wound responses and this corresponds with the rapid nature of the wound-inducibility of the *StPAT* promoter. An accumulation of LA in response to wounding has been previously demonstrated (Conconi *et al.*, 1996). The LA level in tomato leaves increased significantly half an hour after a wounding event and peaked after one hour before falling off, indicating a transient accumulation of LA. This overlapped with the accumulation of jasmonates in plant tissues, peaking one to several hours after wounding (Creelman *et al.*, 1992; Rietz *et al.*, 2004). Another by-product of PATATIN activity is arachidonic acid, a known potent elicitor of phytoalexins (Bostock *et al.*, 1981; Tonón *et al.*, 2001) which are a vital part of a plant's defence against biotic stress during the hypersensitive response. The action of PATATIN also generates the precursors for the synthesis of toxic fatty acids, which could inhibit microbial invasion (Andrews *et al.*, 1988).

A role for PATATIN in defence against biotic stress is further indicated by the β -1,3 glucanase activity that has been attributed to PATATIN (Tonón *et al.*, 2001). This enzyme activity targets β -1,3 glucans in the cell walls of invading

fungi, restricting hyphal growth. Interestingly, β -1,3 glucanase expression is up-regulated during PPD in cassava storage roots (Cortés *et al.*, 2002; Reilly *et al.*, 2004) and the hypersensitive response (HR) in cassava leaves (Kemp *et al.*, 2005), supporting a role for this enzyme during wounding-related responses.

It should be noted here too that a protein cross-reacting with an antiPATATIN-specific antibody had LAH activity and was found in potato flowers, more specifically anthers and petals (Vancanneyt *et al.*, 1989). It is known that large quantities of esterases are generated in anthers and are involved in pollen development, and that PATATIN has esterase activity. This goes some way to explaining the detection of GusP activity in *StPAT::GusP* reporter lines.

4.3.2 Subcellular localisation of PATATIN as an indicator of function

It appears likely that PATATIN is involved in responding to biotic stress since the activity of the protein has the potential to lead to the synthesis of phytoalexins, MeJa and toxic fatty acids, and the direct effect of the protein can lead to suppressed insect and fungal growth rates. The wound-associated activity of GusP should therefore be unsurprising since biotic stresses involve a wounding event. Still unresolved are the details of how the active GusP protein localises to wounding sites in reporter lines.

Given that there is enough potential LAH activity in potato tubers to hydrolyse their entire lipid content in a few minutes, it is perhaps unsurprising that PATATIN has been reported to be sequestered in lysosomes or vacuoles where it remains inactive (Andrews *et al.*, 1988; Strickland *et al.*, 1995). This is backed up by data from the online Signal Peptide database (SPdb) (Choo *et al.*, 2005). Thirty-four results were generated when challenging the SPdb using 'patatin' as the search term. All PATATIN precursor sequences returned were reported to have a vacuole-targeting signal peptide of either eleven or twenty-three amino acids. Compartmentalisation of PATATIN would allow it to be rapidly released on wounding, although this has never been demonstrated. While this theory is sound, it is not applicable to the reporter lines generated in this study since the signal peptide is associated with the PATATIN protein sequence rather than the promoter sequence and would not be present in the GusP reporter gene. Indeed, submitting the GusP protein sequence to an online signal peptide predictor, SignalP 3.0, yielded a probability of zero that the sequence contains a signal peptide (Bendtsen *et al.*, 2004). This, together with the widespread use of β -

glucuronidases as reporter proteins, indicates it is unlikely that GusP is sequestered in vacuoles or lysosomes in the reporter lines used here.

4.3.3 Expression of *GusP* in reporter lines in response to wounding

It is improbable that GusP is sequestered within organelles and released on wounding. It is also doubtful that GusP is present throughout the plant but only activated on wounding since GusP is detectable in unwounded roots without the need for activation. Therefore, the most likely method for local GusP accumulation around wound sites is through up-regulation of *GusP* transcription.

Semi-quantitative PCR (sqPCR) was unable to detect a considerable increase in *GusP* expression at both half an hour and two hours after a wounding treatment (the expression level seen in unwounded leaves should be due to expression at hydathodes). This initially seems contradictory given the increase in GusP protein around wound sites. However, several explanations are available for this apparent paradox.

It is possible that there is an increase in *GusP* expression driven by the *StPAT* promoter, but that the increase is rapid and transient. Within the thirty minute period after wounding but before RNA extraction, the expression may have peaked and then fallen again to the basal rate. As such, sampling the *GusP* expression level thirty minutes after wounding would not be able to detect the wound-associated change in expression. The activity of plant PATATIN-related proteins is frequently up-regulated rapidly following stress (Rietz *et al.*, 2004), although the nature of this activity is normally not transient.

Alternatively, the very slight increase in *GusP* expression may be all that is necessary to bring about the associated change in GusP staining. Only a small percentage of the cells in a leaf stain positive for GusP since the wound response in this case is highly localised around the wound site (estimated 2% of cells by area show positive staining in response to a single cut). As the *GusP* expression level is sampled in whole leaves, the effect seen around wound sites is diluted by the majority of leaf cells that do not respond to the wounding event. Additionally, only a relatively small increase in GusP is necessary to bring about a much larger increase in staining since, once synthesised, GusP protein is stable for at least a few days (Weinmann *et al.*, 1994). Perhaps only a very small change in *GusP* expression should be expected.

A third explanation concerns the turnover of mRNA. It is possible that *GusP* mRNA is being transcribed in response to wounding, but is then degraded or destabilised rapidly. This would still allow a small quantity of wound-associated GusP protein to be synthesised, but the mRNA level would not appear to increase by the same factor. mRNA stability is increasingly recognised as an important factor in gene regulation (Gutiérrez *et al.*, 1999; Belostotsky, 2008). It significantly affects the expression level of 53% of stress regulated genes (Fan *et al.*, 2002) and approximately 1% of *Arabidopsis* mRNAs are classified as unstable (half-life less than sixty minutes) (Gutiérrez *et al.*, 2002). Some mammalian transcripts have a half-life as short as eight minutes (Zhang *et al.*, 1993). A comparison of stressed and non-stressed carcinoma cell lines revealed that the transcription level of *RIBOSE 5-PHOSPHATE ISOMERASE A (RPIA)* increased 1.35-fold after a heat shock treatment (Fan *et al.*, 2002). The *RPIA* mRNA level however was 0.45-fold lower in stressed cells than in non-stressed cells, indicating that the quantity of *RPIA* mRNA did not reflect the transcriptional level of this gene. Transcripts of *PvPRP1* in cultured bean cells decreased to 6% of their original level four hours after treatment with a fungal elicitor while the transcription rate remained unchanged (Zhang *et al.*, 1993). Furthermore, a cDNA microarray approach demonstrated that 34% of mechanical stress (touch) induced transcripts were unstable and that many of these were expressed at higher levels in roots than flowers (Gutiérrez *et al.*, 2002). These mechanical stress-induced transcripts were detected within minutes of wounding and then very rapidly decreased thereafter. It was stated in this study that “mRNA stability is an important regulatory component of the touch response” (Gutiérrez *et al.*, 2002). To date, evidence has not been provided showing instability of any β -glucuronidase transcripts, although mRNA stability can be determined by sequence elements in the 3' UTR of a transcript and so may be specific to the cloning vector rather than the transgene (Gutiérrez *et al.*, 2002). Multiple AU-rich elements (AREs, RNA sequence AUUUA) in the 3' UTR are proposed to contribute towards the destabilisation of transcripts (Shaw & Kamen, 1986). Two AREs are located in close proximity in the *NOS* polyA signal downstream of *GusP* in the cloning vector used here. However, given the common use of this particular polyA signal in cloning vectors, it is improbable that it causes transgene mRNA destabilisation.

Whatever the reason for the discrepancy between mRNA and protein levels of the reporter gene, it seems easier to justify the increase in staining as having

derived from an increase in *GusP* transcription rather than from activation of *GusP*. This indicates sequence elements within the *StPAT* promoter might be responsible for wound-inducible activity.

4.3.4 The relationship between *StPAT* promoter sequence and activity

The regulation of a gene largely depends on transcription factor binding sites, or *cis* regulatory motifs, within its promoter region. Unfortunately, gene regulation via *cis* motifs is poorly understood in plants in comparison to the genes themselves. Pinpointing motifs or even regions important in promoter activity can be difficult given the complex nature of gene signalling *in planta*. However, identifying potential *cis* motifs in the *StPAT* promoter could aid in determining the mechanisms through which the promoter is activated during a wound response. It could also help explain the other aberrant aspects of *StPAT* promoter-derived *GusP* activity.

Alignment of the *StPAT* promoter with other class I *PATATIN* promoters revealed the *StPAT* promoter was missing forty-five base pairs from the 3' end. This included the native TATA box and the region harbouring a twenty-two base pair sequence whose absence would have unambiguously classified the *StPAT* promoter as a class I rather than a class II *PATATIN* promoter. The absence of the native TATA box appeared to be compensated for by other TATA boxes since *GusP* staining was strong in the majority of lines. The deletion of the 3' forty-five base pairs from the *StPAT* promoter could be responsible for the aberrant promoter activities (i.e. wound-inducibility, activity in anthers and multiple hydathodes) seen in reporter lines. The forty-two base pairs of Gateway[®]-associated sequence was probably not responsible for the wound-inducible nature of the *StPAT* promoter, although direct fusions between the *StPAT* promoter and *GusP* would confirm this.

The presence of *cis* elements within the remaining *StPAT* promoter sequence (i.e. that used in this study) could also be responsible for wound-inducibility. Promoter deletion analysis demonstrated that the 3' 261 bp of the *StPAT* promoter was sufficient to confer the activity in both *Arabidopsis* and tobacco. Within this region, a large number of *cis* regulatory motifs potentially associated with responding to stress were identified, a number of which were also found in the wound-responsive *SPORAMIN* promoter. A different class I *PATATIN* promoter, the *StB33* promoter, was not wound-inducible and although its

sequence was highly homologous to that of the *StPAT* promoter over the 3' 261 bp, conflicts between the two were found. Importantly, one conflict led to the deletion of a wound response-associated PI IIK motif (Palm *et al.*, 1990) in the *StB33* promoter, which highlighted its potential significance in conferring wound-inducibility on the *StPAT* promoter. The comparison of the sequence of the *StPAT* promoter with five other wound-inducible plant promoters identified seven highly conserved sequence motifs, four of which were present in the important 3' 261 bp of the promoter essential for wound-inducibility.

At this juncture the association between identified *cis* motifs and promoter activity may be merely coincidental. To directly link one or a group of *cis* elements to the wound-inducible activity of the *StPAT* promoter, considerable further work is required.

4.3.5 PATATIN-like proteins

Since the role of PATATIN in potato tubers is not fully understood, it is useful to examine the role of PATATIN-like proteins in other plant species. Sequencing of the *Arabidopsis* genome has facilitated the characterisation of the *PATATIN*-like gene family in the model species (Matos *et al.*, 2008), while some studies have also been carried out on tobacco PATATIN-like proteins.

PATATIN-like genes in *Arabidopsis* constitute a small family. Sequence comparison between potato *PATATIN* genes and the *Arabidopsis* genome yielded nine gene sequences in *Arabidopsis* designated as *PATATIN-LIKE PROTEIN* (*AtPLP1-9*) (La Camera *et al.*, 2005). Other publications abbreviate the same genes as *AtPAT* or *AtPLA*, although *AtPLP* will be used here throughout for clarity. Of these nine genes, *AtPLP2* is of particular interest since it has been reported as being stress-responsive. *AtPLP2* expression is developmentally controlled in *Arabidopsis* (Rietz *et al.*, 2004), unlike the root- or tuber-specificity of *PATATIN* in potato, cassava, tobacco and *Arabidopsis*. The *AtPLP2* promoter, when fused to the *E. coli* β -*GLUCURONIDASE* (*uidA*) reporter gene, conferred GUS activity in response to MeJa, ACC, wounding, pathogens and nutrient deficiency (Rietz *et al.*, 2004). The broad stress response of *AtPLP2* typically occurs on the hours/days timescale, reminiscent of responses to pathogens rather than a wounding event (minutes timescale).

A further publication could not replicate induction of the *AtPLP2* promoter by mechanical stress, although treatment of leaves with ACC and MeJa combined did induce the promoter. *AtPLP2* protein was not detected in wounded *Arabidopsis* leaves up to forty-eight hours after a wounding event and *AtPLP2::GUS* reporter lines showed no GUS staining after severing of leaves (La Camera *et al.*, 2005). However, reporter lines did stain positive for GUS at multiple hydathodes, mirroring the background leaf expression of *StPAT::GusP* reporter lines. *AtPLP2* was also confirmed as being responsive to pathogen inoculation – a drop of *Botrytis cinerea* spores placed on leaf tissue led to *AtPLP2* protein accumulation after forty-eight hours and *AtPLP2::GUS* reporter lines showed positive, localised GUS staining around inoculation sites (La Camera *et al.*, 2005). *AtPLP2* accumulation appeared to negatively affect resistance to *B. cinerea*, questioning the evolutionary advantage in conserving this gene. However, *AtPLP2* is able to promote programmed cell death and thereby increase resistance to viruses (La Camera *et al.*, 2009). A more recent study seemed to agree with the earlier results, showing an increase in *AtPLP2* transcripts two hours after a wounding event, remaining above background level four hours after wounding (Matos *et al.*, 2008).

AtPLP2 is a stress responsive gene with phospholipase activity and sequence homology to *PATATIN*. *AtPLP2* is responsive to pathogens and may also be induced by wounding, although this effect has not always been replicated. Additionally, *AtPLP3* is drought-responsive, *AtPLP6* is responsive to pathogens and *AtPLP7* is responsive to both drought and pathogens (La Camera *et al.*, 2005; Matos *et al.*, 2008). Furthermore, it is generally accepted that phospholipase A activity is induced by one or a combination of three signals; auxin, wounding or pathogens (Rietz *et al.*, 2004). These results support the theory that *S. tuberosum* *PATATIN* may play a role in responding to stress.

Tobacco *PATATIN*-like phospholipases respond to pathogens. Phospholipase activity increased in tobacco after tobacco mosaic virus infection and preceded an accumulation of a jasmonic acid precursor, 12-oxophytodienoic acid (OPDA) (Dhondt *et al.*, 2000). This phospholipase activity was shown to arise from *PATATIN*-like proteins, designated NtPAT. Furthermore, the regulation of phospholipase activity mirrored that of lipoxygenase, an enzyme in the jasmonic acid synthesis pathway (Dhondt *et al.*, 2002), linking the action of *PATATIN* to jasmonic acid generation. In addition to the tobacco proteins, a *PATATIN*-LIKE protein (VuPAT1) was isolated from cowpea and had LAH activity (Matos *et al.*,

2001). The *VuPAT1* promoter contains drought-responsive sequence motifs and *VuPAT1* mRNA levels increase after drought stress.

The evolution of *PATATIN-LIKE* families in plants indicates these genes have important functions. Enzymatic PATATIN-like proteins in plant species other than potato appear to be regulated by wound hormones, pathogens and drought. While *PATATIN* genes in potato have not previously been shown to be stress-responsive, a role in defence has often been inferred. Here, rapid, localised wound-responsiveness of the *StPAT* promoter supports those inferences and reveals the first instance of a stress-responsive potato *PATATIN* gene. The various enzyme activities of PATATIN make predicting its role in the stress response difficult, although multiple roles should not be discounted.

4.3.6 Future work

The activity of the *StPAT* promoter appears to be wound-inducible. It was unexpected then that the level of *GusP* transcripts under the control of the *StPAT* promoter did not increase after a wounding treatment. To understand this inconsistency, the activity of *GusP* should be assessed in leaves of *Arabidopsis* plants carrying the *StPAT::GusP* cassette before and after wounding. A quantitative *GusP* assay is accessible that involves incubation of plant extracts with the substrate 4-methylumbelliferyl β -D-glucuronide (4-MUG). Hydrolysis of 4-MUG by *GusP* produces the fluorochrome 4-methyl umbelliferone (4-MU), which can be measured and compared against β -glucuronidase standards.

It is possible that the expression of *GusP* under the control of the *StPAT* promoter increases after wounding, but only transiently for a very short period of time. *GusP* reporter visualisation is ill-suited to studying temporal promoter expression patterns since the stain is cumulative over time. Another reporter expression plasmid should be synthesised replacing *GusP* with a *GFP* reporter gene. This would facilitate investigation on the temporal expression derived from the *StPAT* promoter since changes in expression can be observed over time using GFP.

Further promoter deletions would help to pin-point the *StPAT* promoter *cis*-regulatory motif(s) responsible for wound-inducible expression. Currently, it is known that a 261 base pair region is sufficient to confer wound-inducible expression, although numerous potential wound-responsive *cis* motifs are located

within this region. The creation of synthetic versions of the 3' 261 bp of the *StPAT* promoter with small sequence differences would facilitate this search.

Finally, the reporter expression plasmids should be used to transform potato plants. Wounding assays in transgenic lines would demonstrate whether this *PATATIN* promoter version has a role in responding to wounding in the species from which it was isolated.

5 OVER-EXPRESSION OF ANTIOXIDANT GENES IN *ARABIDOPSIS*

5.1 Introduction

5.1.1 A strategy for increasing resistance to oxidative stress

PPD in cassava is generally accepted to be the result of severe, prolonged oxidative stress resulting from an initial oxidative burst that is not sufficiently reversed. Oxidative stress is defined as an imbalance between pro-oxidative and anti-oxidative cellular reactions (Bartosz, 1997). It is ultimately caused by an increase in the generation of reactive oxygen species (ROS) within cells in response to stress. ROS are vital stress signalling molecules but at high concentrations are capable of oxidising all the components of plant cells, leading to cell damage or death. Oxidative stress is an unavoidable consequence of all forms of stress, biotic and abiotic, and is responsible for billions of dollars of crop losses annually (Mittler, 2006).

Plants, for the most part, are sessile organisms. As such, they possess a limited number of strategies to avoid oxidative stress. It is not surprising then that plants have evolved complex pathways for tolerating oxidative stress. This tolerance relies on a balance between ROS production and removal and involves coordinated interplay between large suites of genes. ROS removal systems rely on antioxidant molecules that can efficiently scavenge ROS. The relative contributions of different antioxidant molecules to scavenging ROS during stress is unknown, although antioxidant enzymes certainly play a major role in this process (Mittler, 2002). Genes encoding three antioxidant enzymes have been isolated from cassava and their sequences published: *ASCORBATE PEROXIDASE 2* (*MecAPX2*), *CATALASE 1* (*MecCAT1*) and *SUPEROXIDE DISMUTASE 2* (*MecSOD2*). These genes therefore provide attractive targets for a biotechnological approach to extend the shelf life of cassava storage roots through increased ROS scavenging.

5.1.2 APX

ASCORBATE PEROXIDASE (APX, L-ascorbate:H₂O₂ oxidoreductase, EC 1.11.1.11) is found in the majority of cellular sites in plants and acts to detoxify H₂O₂ as part of the ascorbate-glutathione cycle, utilising ascorbate as the specific electron donor (Asada, 1999; Mittler & Poulos, 2005). The high affinity of APX for H₂O₂ makes it efficient at regulating H₂O₂ levels and indicates a role in the fine-tuning of ROS rather than mass detoxification (Mittler, 2002; Hong *et al.*, 2007). Most plant species possess multiple APX isozymes, categorised according to their subcellular localisation. While cassava has been predicted to contain many PEROXIDASE (POX) isozymes, to date only one APX gene, cytosolic *MecAPX2*, has been identified (Gómez-Vásquez *et al.*, 2004; Passardi *et al.*, 2007). It should be noted that the GenBank database also contains an accession for *MecAPX3* (AY973623), although its sequence is identical to that of *MecAPX2* and so both accessions are assumed to refer to the same gene. Cytosolic forms of APX (cAPX) are generally considered the most responsive to environmental stimuli (Ishikawa & Shigeoka, 2008).

MecAPX2 expression is transiently up-regulated after harvesting in cassava storage roots. Microarray analysis demonstrated that transcripts of this gene had increased 1.70-fold twenty-four hours after harvesting, whereupon they subsequently returned to pre-harvest levels (Reilly *et al.*, 2007). The total POX activity of storage root tissue also experiences a transient increase after stress treatment in cassava. A yeast elicitor was able to trigger a peak four-fold induction of POX activity forty-eight hours after inoculation of a cassava cell suspension (Gómez-Vásquez *et al.*, 2004), with POX activity remaining low before and after this peak. Wounding of storage roots meanwhile resulted in a transient increase in POX twenty-four hours after harvesting (Isamah, 2004). It seems likely then that the induction of APX in cassava storage roots in response to wounding is insufficient in timing or magnitude to counteract the high dose of ROS the cells receive.

Previous studies have served as proof-of-concept that resistance to oxidative stress can be modulated by altering APX expression levels in plant tissues. Transgenic tobacco plants over-expressing a pea cAPX in all tissues demonstrated an increase in resistance to MV, chilling and high-light stress (Allen *et al.*, 1997). Furthermore, the constitutive over-expression of pepper ASCORBATE PEROXIDASE 1-LIKE (*CaPOA1*) in tobacco resulted in plants with increased tolerance to MV and a fungal pathogen (Sarowar *et al.*, 2005) while

Arabidopsis plants expressing an antisense copy of thylakoidal APX (*tAPX*) showed a 50% reduction in *tAPX* activity and were more sensitive to MV-induced oxidative stress (Tarantino *et al.*, 2005). This information supports the notion that storage root-specific *MecAPX2* over-expression is a viable strategy for modulating the oxidative stress phenomenon of PPD in cassava.

5.1.3 CAT

CATALASE (CAT, H₂O₂:H₂O₂ oxidoreductase, EC 1.11.1.6) is ubiquitous in all multicellular organisms and like APX, acts to detoxify H₂O₂ (Feierabend, 2005). CAT does not consume reducing power and is capable of scavenging large quantities of H₂O₂ due to its high rate of reaction. However, it also has a low affinity for H₂O₂ and the majority of isozymes are predominantly compartmentalised within peroxisomes, making CAT of limited use in preventing oxidative damage across the whole plant cell (Mohamed *et al.*, 2003; Feierabend, 2005). In most well characterised plant species, three CAT gene family members have been isolated (Reilly *et al.*, 2001). In cassava only one full-length CAT gene, peroxisomal *MecCAT1*, has been isolated to date, although Southern blot analysis indicated the presence of a small gene family with at least two or three members (Reilly *et al.*, 2001).

MecCAT1 expression increased in storage roots after harvesting. This increase was higher in a cultivar with low PPD susceptibility (TMS 30572) than a susceptible cultivar (MCOL22), suggesting a positive correlation between CAT activity of storage roots and resistance to PPD in cassava (Reilly *et al.*, 2001). Furthermore, *MecCAT1* appeared to be largely storage root-specific, since *MecCAT1* mRNA was barely detectable in leaf tissue. Localisation of CAT activity also demonstrated that TMS 30572 storage roots maintained a high level of activity for six days after harvesting, while the activity in MCOL22 storage roots decreased dramatically four days after harvesting and remained low (Reilly *et al.*, 2001), further supporting an association between CAT and PPD resistance.

The expression of CAT genes has been modulated to successfully overcome oxidative stress-related phenomena in species other than cassava. Constitutive over-expression of a wheat CAT gene in rice protected transgenic plants from wilting induced by low temperature stress. During the chilling stress, CAT activity remained at least three-fold higher in transgenic plants than controls, which helped to maintain a significantly lower H₂O₂ level throughout (Matsumura *et*

et al., 2002). The introduction of a bacterial *CAT* gene (*KatE*) into tomato plants increased *CAT* activity by three-fold and conferred protection against MV-induced photo-oxidative damage, as well as some protection against drought and chilling stress (Mohamed *et al.*, 2003). Findings similar to these have been reproduced elsewhere (Polidoros *et al.*, 2001; Vandenabeele *et al.*, 2004) indicating that *CAT* over-expression in storage roots could increase resistance to ROS-mediated PPD in cassava.

5.1.4 SOD

SUPEROXIDE DISMUTASE (SOD, superoxide:superoxide oxidoreductase, EC 1.15.1.1) constitutes the initial line of cellular defence against oxidative stress, detoxifying superoxide anions (O_2^-) and releasing H_2O_2 as a by-product (Lee *et al.*, 1999). SOD enzymes are classified into three groups according to their metal cofactor: copper/zinc SOD (Cu/ZnSOD, localised to chloroplasts, the apoplast and the cytosol), manganese SOD (MnSOD, mitochondria and peroxisomes) and iron SOD (FeSOD, chloroplasts) (Kim *et al.*, 2008). Plant genome sequence data supports the existence of small families of SOD genes. To date two *Cu/ZnSOD* genes have been isolated in cassava – *MecSOD1* and *MecSOD2* (Lee *et al.*, 1999; Shin *et al.*, 2005).

Expression of *MecSOD1* is high in tuberous roots and is inducible by a variety of stresses including heat, wounding and MV treatment (Lee *et al.*, 1999). *MecSOD2* is expressed most highly in stem tissue but is similarly up-regulated in response to stresses (Shin *et al.*, 2005). MnSOD activity has been detected in cassava implying the existence of as yet unidentified SOD genes (Isamah *et al.*, 2003). Interestingly, the total SOD activity of cassava storage root tissue does not increase significantly after harvesting, although the contribution from Cu/ZnSOD activity may do so (Isamah *et al.*, 2003). There is an initial decline in total SOD activity during the first twenty-four hours post-harvest, followed by a slight increase over the next forty-eight hours (Reilly *et al.*, 2004), a response that is insufficient in terms of delaying PPD.

The over-expression of SOD genes to alleviate oxidative stress has been carried out previously in other plant systems. A chloroplastic *Cu/ZnSOD* from pea was over-expressed in tobacco plants, permitting the maintenance of a higher photosynthetic rate than wild-type plants. Furthermore, these transgenic plants were significantly more resistant to MV-induced oxidative damage (Gupta *et al.*,

1993a). Interestingly, the increase in SOD activity observed in these plants caused an increase in APX activity of a similar magnitude (Gupta *et al.*, 1993b). Other investigations have yielded similar results concerning resistance to oxidative stress (Slooten *et al.*, 1995; Van Camp *et al.*, 1996), but to date no studies involving root-specific over-expression of SOD have been conducted in plants. *MecSOD2* is an attractive target for over-expression studies in cassava storage roots, due to its relatively low level of native expression in this tissue and the insufficient activity of SOD after harvesting of wild-type plants. Suggestions that SOD might induce H₂O₂ scavenging enzymes are encouraging but expected, since the activity of SOD generates H₂O₂ as a by-product.

5.1.5 *Arabidopsis* as a model for studying cassava PPD

The aim of this research is to over-express three genes encoding ROS scavenging enzymes, *MecAPX2*, *MecCAT1* and *MecSOD2*, in cassava storage root tissue in order to modulate the PPD response. However, studies including genetic engineering of cassava are demanding and time-consuming since this crop species is challenging to transform. Additionally, cassava has a nine month growth cycle and the production of glasshouse-derived storage roots is unpredictable. The manipulation of the plant in the laboratory is also difficult due to its large size.

It was deemed prudent therefore to transform a model plant species prior to the transformation of cassava. This would reveal whether the expression plasmids containing the target genes were functional and able to confer transgene expression *in planta*. It would also provide an indication of the expected level of transgene expression after transformation of cassava, as well as the degree of resistance that transgenic plants might show to oxidative stress. *Arabidopsis thaliana* was chosen as the model plant for this research since this species is readily transformable and has a short growth cycle of 8 weeks. Furthermore, its small genome size (approximately 25,000 genes) permits efficient genetic manipulation. Its small size and simple growth requirements make laboratory growth of the plant very simple while the publication of the entire *Arabidopsis* genome sequence has facilitated biotechnological investigation using this species (Koornneef & Scheres, 2001; Somerville & Koornneef, 2002).

5.1.6 Research aims

This chapter is concerned with the production and analysis of transgenic plants over-expressing genes encoding antioxidant enzymes in *Arabidopsis* roots. The largely root-specific *StPAT* promoter, characterised in chapter 4, was used to drive the expression of the target genes. The transgene expression level was measured in single-insertion, homozygous lines. The impact of transgene expression on antioxidant enzyme activity was then assessed, with promising candidate lines assayed for their resistance to oxidative stress using a range of techniques.

5.2 Results – *MecAPX2*

5.2.1 *Arabidopsis* transformation and selection of transgenic lines

Arabidopsis was subjected to a floral dip transformation by immersing buds in *A. tumefaciens* containing the pDEST™ *MecAPX2* plasmid. Screening for positive transformants in the first generation after transformation (T₁) yielded seventeen transgenic lines. All seventeen plants were confirmed as positive via gDNA PCR genotyping using primer pair 19 (Figure 5.1).

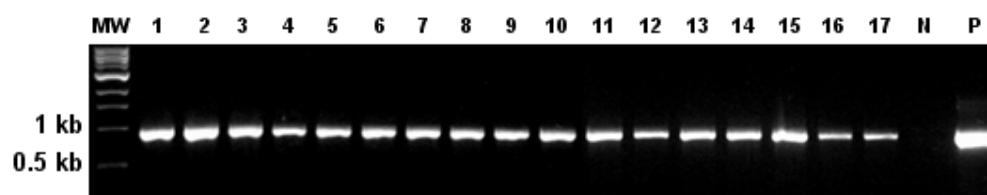


Figure 5.1: Transgenic seedlings identified through a hygromycin B screen are confirmed as harbouring the *StPAT::APX2* cassette using PCR genotyping. Agarose gel image showing PCR products amplified from gDNA of seventeen plants (labelled 1 – 17). MW = molecular weight marker with relevant sizes to the left of the gel image. N = negative control (wild-type gDNA), P = positive control (pDEST™ *MecAPX2* plasmid DNA as template). Expected product size is 952 bp.

Eight of those seventeen lines were identified as having a single insertion (T₂ generation) and subsequently homozygous lines (T₃ generation) were found for seven of those eight single-insertion lines. Three of these lines were randomly chosen and carried forward for use in the subsequent experiments. These lines were referred to as APX 1.10, APX 4.2 and APX 6.2 and the transgenic cassette referred to as *StPAT::APX2*.

5.2.2 *Arabidopsis* harbouring the *StPAT::APX2* cassette over-express the transgene in root tissue

The three transgenic lines chosen (APX 1.10, APX 4.2 and APX 6.2) were analysed to assess the level of *MecAPX2* expression. Plants were grown on MS media plates stood 5° from vertical to facilitate harvesting of root tissue. After 10 days growth, RNA was extracted from the root tissue of a batch of approximately

eighty plants per line. After cDNA had been synthesised from RNA, sqPCR was used to determine the relative level of cassava *APX2* transcript in wild-type and transgenic root tissue. The relative abundance of the total *APX* transcript level was also determined to establish whether the over-expression of one *APX* gene was able to significantly impact on the total *APX* expression level. Lastly, the total *CAT* and *SOD* transcript levels were measured to assess whether over-expression of one antioxidant gene affected the expression of others.

The sqPCR results (Figure 5.2) demonstrated that transgenic plants harbouring the *StPAT::APX2* cassette were over-expressing the transgene in root tissue, while no transgene expression was detectable in wild-type root tissue. The three independent lines showed varying levels of transgene expression, with line 4.2 expressing most highly. The over-expression of *MecAPX2* appeared to increase total *APX* expression from the wild-type level to some extent, particularly in line 4.2. The total *CAT* and total *SOD* expression levels were similar in wild-type and transgenic lines. The direct comparison of the intensity of products from different sqPCR runs (i.e. different rows in Figure 5.2) should be avoided since factors associated with amplification (for example, primer binding specificity) could not be normalised across all runs.

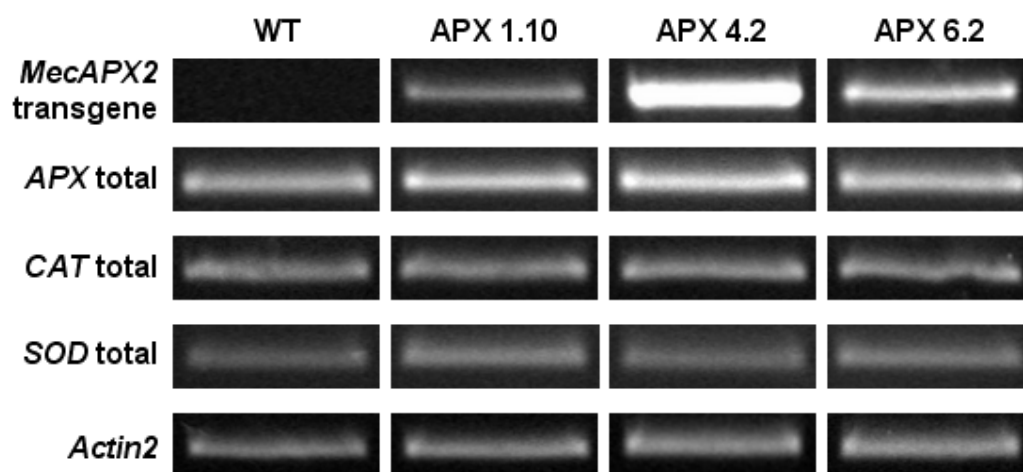


Figure 5.2: Transgenic *Arabidopsis* lines harbouring the *StPAT::APX2* cassette show varying levels of transgene expression in root tissue. Agarose gel images from sqPCR analysis. Number of amplification cycles: *MecAPX2* – 28; *APX* total – 21; *CAT* total – 23; *SOD* total – 27; *Actin2* – 21. Primer pairs: *MecAPX2* – 20; *APX* total – 21; *CAT* total – 22; *SOD* total – 23; *Actin2* – 24. WT = wild-type, Col-0.

5.2.3 *Arabidopsis* harbouring the *StPAT::APX2* cassette have increased APX activity in root tissue

While over-expression of the *MecAPX2* transgene in *Arabidopsis* root tissue was a successful result, an increase in the level of APX enzyme was the ultimate goal. A number of post-transcriptional and post-translational mechanisms could potentially act to dampen the effect of an increase in transcript level. As such, the APX activity of wild-type and transgenic *Arabidopsis* root protein extracts was measured. The APX activity of leaf tissue was assessed independently of the root samples to establish whether the promoter was driving transgene expression in a root-specific manner. Plants were grown for 10 days on MS media stood 5° from vertical to allow rapid harvesting of leaf and root material. Root material from approximately eighty plants was processed for each line. It should be noted here that the assay used assessed the level of both APX and guaiacol peroxidase (GuPX) since both enzymes were capable of using guaiacol as an electron donor (Mehlhorn *et al.*, 1996).

The wild-type activity of APX was significantly higher in root tissue than in leaf tissue (Student's *t*-test: WT leaf vs. WT root, $p < 0.001$) (Figure 5.3). Importantly, the root activity of APX in the three transgenic lines was higher than in wild-type, with lines 4.2 and 6.2 significantly higher (Student's *t*-test: WT vs. 1.10, $p = 0.055$; WT vs. 4.2, $p = 0.017$; WT vs. 6.2, $p < 0.001$). Interestingly, although line 4.2 showed the highest level of transgene and total APX expression in root tissue (Figure 5.2), of the three transgenic lines it possessed the lowest root APX activity. Although it is clear from the graph that the APX level had increased considerably in line 1.10 roots compared to wild-type roots (3.51-fold), it was deemed insignificant by the statistical analysis. This result may have arisen from the small sample number as only three biological replicates were performed for each line. Unfortunately, it was not possible to perform more replicates because of the time needed to grow, process and analyse the plant material.

A Student's *t*-test was performed (WT vs. 1.10, $p = 0.991$; WT vs. 4.2, $p = 0.874$; WT vs. 6.2, $p = 0.926$) showing there was no significant difference in the APX level between wild-type and transgenic leaf samples. This indicated the *StPAT* promoter was driving transgene expression specifically in root-tissue in these transgenic lines.

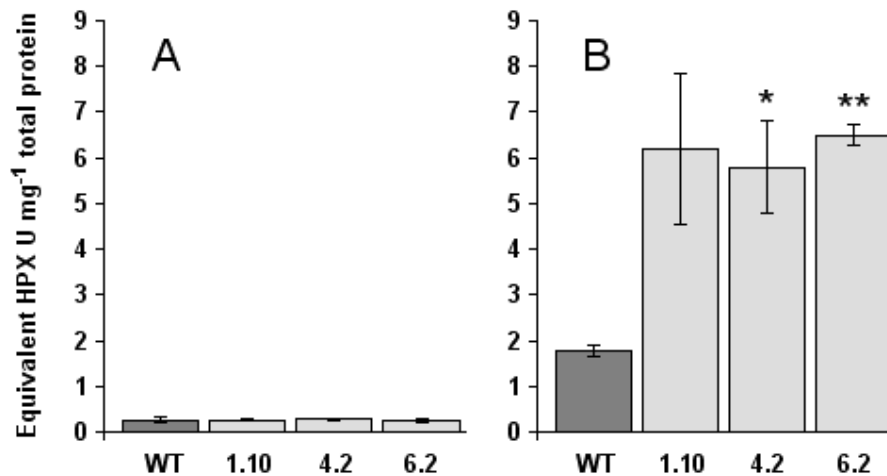


Figure 5.3: Transgenic *Arabidopsis* lines harbouring the *StPAT::APX2* cassette have increased APX activity in root tissue. Mean APX activity is given in HPX equivalents for *Arabidopsis* leaf (A) and root (B) tissue. 1.10, 4.2 and 6.2 refer to independent transformation lines harbouring *StPAT::APX2*, WT = wild-type, Col-0. Asterisks denote enzyme activities significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent ± standard error of the mean (SEM), n = 3.

5.2.4 An increase in APX activity in roots does not confer resistance to abiotic stress

APX directly acts on H₂O₂, a reactive oxygen species (ROS) involved in stress signalling that can also damage cells at high concentrations. Since plants of the three transgenic lines had a higher APX activity than wild-type plants, they were assessed to examine if this conferred resistance to oxidative stress. Seed was plated onto MS media containing a variety of agents that generate oxidative stress and the plants grown for 7 days. Photographs of the plates were taken and a visual assessment of the stress resistance of each line was made. By performing a small scale experiment, it was possible to subject the plants to a broad range of stresses in a short period of time and quickly establish which, if any stresses the plants had resistance to.

By examining the unstressed plants (Figure 5.4A), it was observed that seedlings of lines 4.2 and 6.2 were considerably smaller than both wild-type and line 1.10 and this was taken into account when analysing the stressed seedlings. When the lines were grown on MS media whose pH had been decreased from 5.8

to 3.8, all lines appeared equally affected in terms of size, although wild-type plants suffered more discolouration of leaf tissue (Figure 5.4B). When the pH of the media was increased to 7.8, APX 6.2 seedlings were of a similar size to wild-type indicating that they had some resistance to the high pH since they were smaller under non-stress conditions (Figure 5.4C). Wild-type and transgenic seedlings on MS media with H₂O₂ appeared consistently affected by the stress at 40 µM and 200 µM, while lines 4.2 and 6.2 on 1,000µM H₂O₂ were considerably smaller than seedlings of line 1.10 (Figure 5.4D – F). Discolouration of leaf tissue was seen in all lines at 200 µM, but no discolouration was seen at either 40 µM or 1,000 µM. When plated on MS media with either NaCl (Figure 5.4G – I) or MV (Figure 5.4J – L), transgenic seedlings showed no obvious signs of resistance to the chemical stressing agents compared to wild-type.

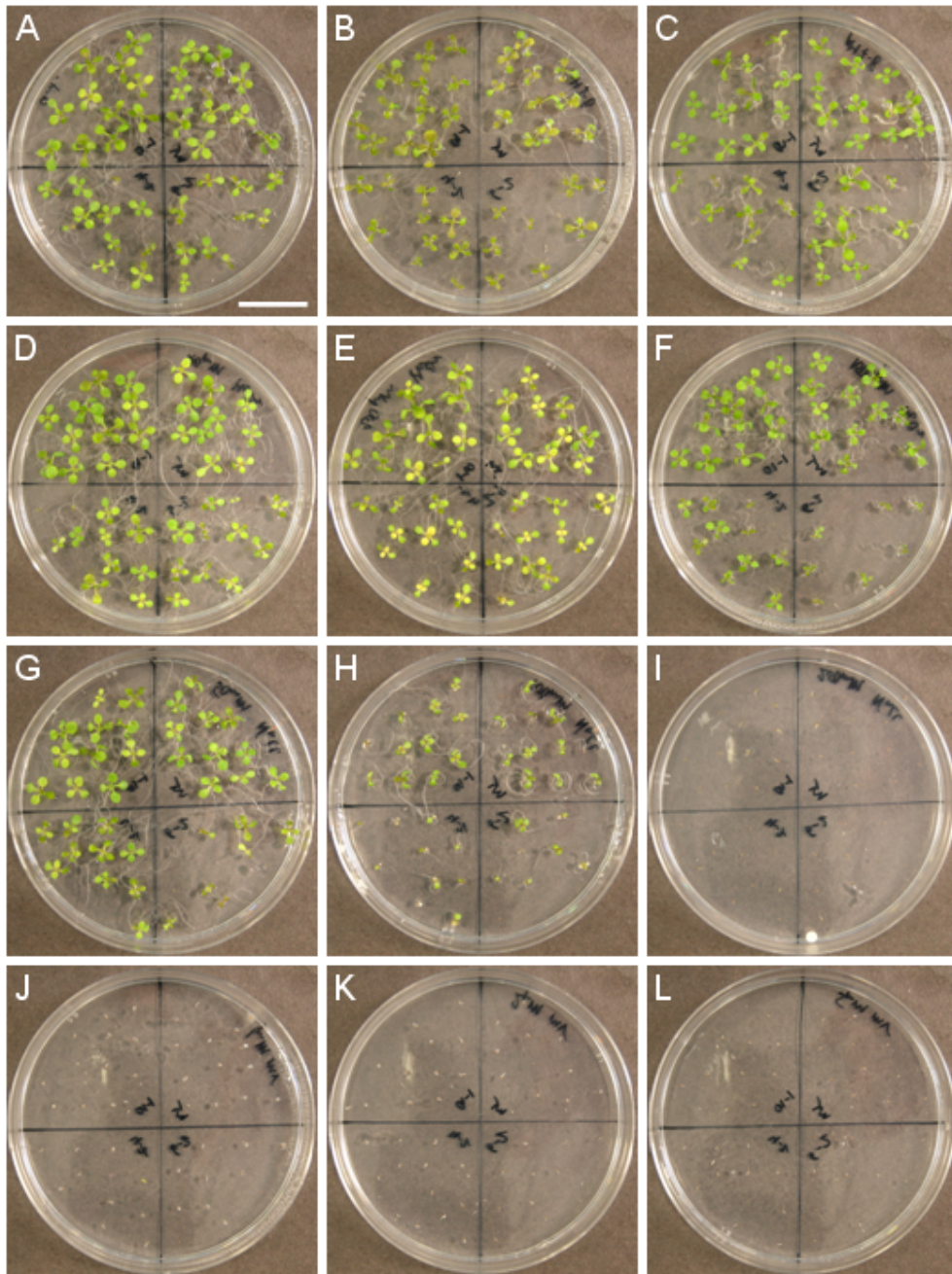


Figure 5.4: Transgenic *Arabidopsis* lines harbouring the *StPAT::APX2* cassette are not more stress resistant than wild-type plants. On each Petri dish: wild-type (top right); APX 1.10 (top left); APX 4.2 (bottom left); and APX 6.2 (bottom right). Seeds were germinated and grown on MS media containing stress chemicals: (A) control; (B) media at pH 3.8; (C) media at pH 7.8; (D) 40 μM H_2O_2 ; (E) 200 μM H_2O_2 ; (F) 1,000 μM H_2O_2 ; (G) 50 mM NaCl; (H) 100 mM NaCl; (I) 200 mM NaCl; (J) 1 μM MV; (K) 2 μM MV; and (L) 5 μM MV. Bar represents 2 cm, all photographs taken at the same magnification.

Transgenic lines over-expressing *MecAPX2* did not show substantial resistance to chemical-based mimics of oxidative stress. The same lines were then subjected to a more realistic environmental stress. Wild-type and transgenic plants were grown side by side for 23 days. At this point, water was withheld from half of the plants of each line while the remainder were watered normally. After 7 days of drought stress, the plants were photographed and visually assessed.

None of the transgenic lines showed any resistance to the imposed stress (Figure 5.5). While transgenic plants watered normally appeared as healthy as watered wild-type plants, those that had undergone drought stress seemed less healthy than their wild-type counterparts; the primary stem had elongated more in drought stressed wild-type plants than in droughted transgenic lines.

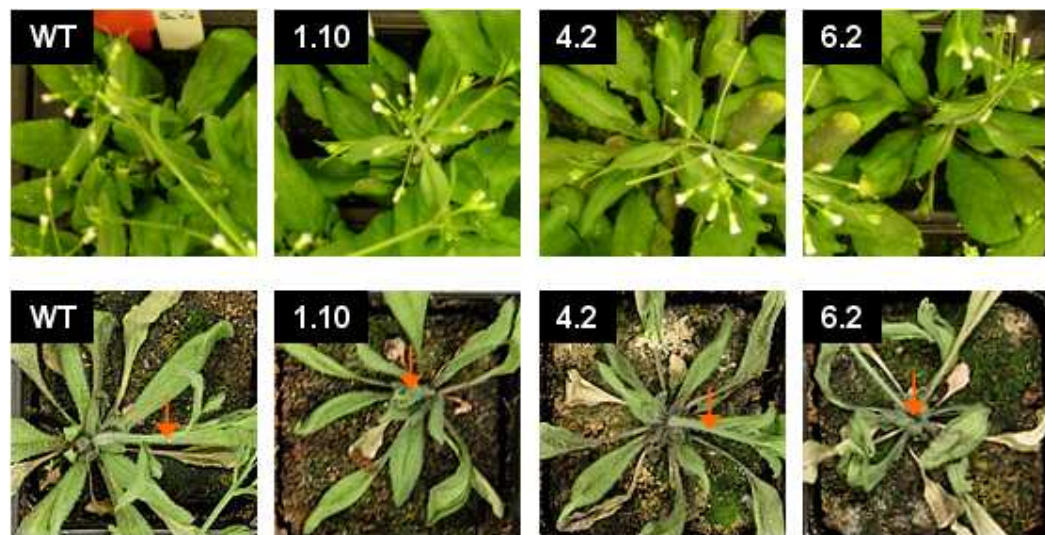


Figure 5.5: Over-expression of *MecAPX2* in *Arabidopsis* roots does not confer resistance to drought. Between 23 and 30 DAG, plants were either provided with ample water (top row) or had water withheld (bottom row). A representative plant from each line was photographed. Arrows indicate position of primary stem.

5.2.5 Quantitative assays suggest over-expression of *MecAPX2* confers some resistance to abiotic stress induced by H₂O₂

While the above stress experiments were useful, they were purely qualitative. To rigorously assess the degree of resistance that over-expression of *MecAPX2* confers, a quantitative method of analysis was designed. Wild-type and transgenic plants were grown on MS media containing varying concentrations of a stressing chemical. Plates were incubated for 5 days, stood 5° from vertical to ensure roots grew along the media surface. After incubation, photographs of the plates were taken and primary root lengths were measured as an indicator of resistance.

The first quantitative stress resistance experiment entailed growing plants on MS media containing H₂O₂ (Figure 5.6). When the stressing agent was absent, roots of wild-type and line 1.10 plants were very similar in length. Roots of lines 4.2 and 6.2 were shorter, with those of 4.2 significantly so (Student's *t*-test: WT vs. 4.2, *p*=0.033). This agreed with earlier data that suggested 4.2 and 6.2 plants were slower to develop than wild-type plants under normal conditions (Figure 5.4A).

When H₂O₂ was present at a low concentration (40 µM), a similar pattern was seen, although roots of 4.2 and 6.2 were now both significantly shorter than wild-type (Student's *t*-test: WT vs. 4.2, *p*=0.001; WT vs. 6.2, *p*=0.003). The root length of wild-type plants had increased when plants were grown on MS media containing 40 µM H₂O₂ compared to 0 µM H₂O₂, indicating that 40 µM was not sufficient to cause stress.

The mean root length of wild-type plants grown on 200 µM H₂O₂ was shorter than the mean root length of wild-type plants grown without H₂O₂. Although this was not significant (Student's *t*-test: WT 0 µM vs. WT 200 µM, *p*=0.069), it showed that this concentration was sufficient to cause stress. Interestingly, at this concentration roots of 1.10 plants were significantly shorter than wild-type roots, while those of lines 4.2 and 6.2 were now similar to wild-type (Student's *t*-test: WT vs. 1.10, *p*=0.040; WT vs. 4.2, *p*=0.516; WT vs. 6.2, *p*=0.931).

At a high concentration of H₂O₂ (1,000 µM), the mean root length of wild-type plants was significantly reduced compared to unstressed wild-type plants (Student's *t*-test: WT 0 µM vs. WT 1,000 µM, *p*<0.001). The mean root lengths of the three transgenic lines were all higher than wild-type, with those of line 6.2 significantly higher to the 99.5% level (Student's *t*-test: WT vs. 6.2, *p*=0.004).

From this data it can be concluded that over-expression of *MecAPX2* in *Arabidopsis* roots does not confer an advantage under normal physiological conditions. However, under conditions of severe oxidative stress generated by H_2O_2 , some resistance is seen in transgenic lines.

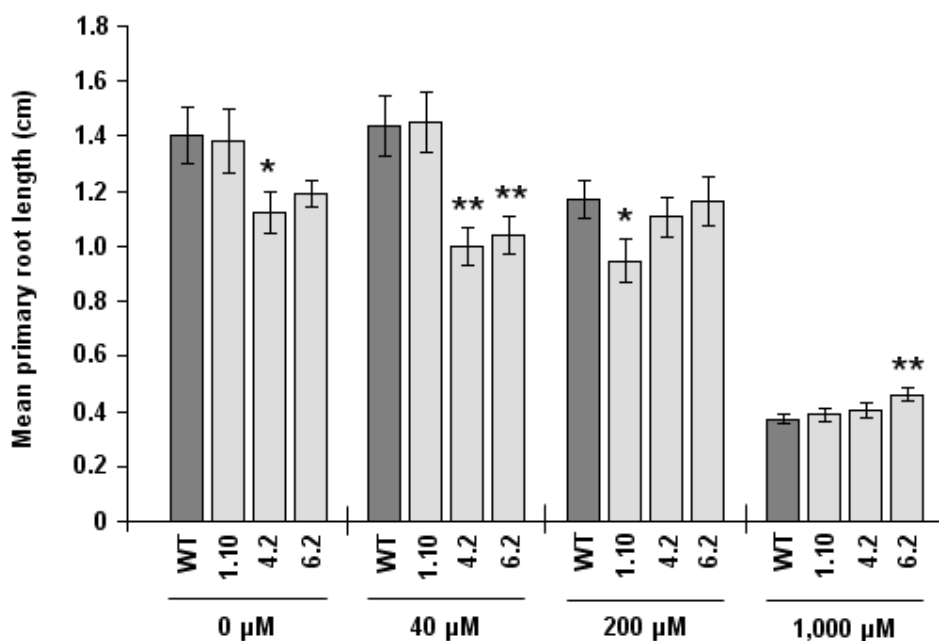


Figure 5.6: Over-expression of *MecAPX2* confers some resistance to high H_2O_2 concentrations on *Arabidopsis* roots. Primary root length of wild-type (WT) plants and three transgenic lines (1.10, 4.2 and 6.2) over-expressing *MecAPX2* in a root-specific manner. H_2O_2 concentration provided at the bottom of the x-axis. Asterisks denote root lengths significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, $18 \leq n \leq 20$.

The above experiment was repeated, this time substituting NaCl into the MS media instead of H_2O_2 (Figure 5.7). NaCl stress, as well as causing osmotic stress and ion toxicity, is known to induce ROS (Hernández *et al.*, 1995) and so an increase in APX activity should alleviate some aspects of salt stress. It is also reasonable to assume that if transgenic lines are more resistant than wild-type, the difference in root length between them would be greater after a longer growth period. As such, the growing time was increased to 10 days.

As expected, a similar pattern of root lengths was seen for the control samples compared to the control samples in the H_2O_2 experiment (Figure 5.6).

Roots of lines 4.2 and 6.2 were both significantly shorter than wild-type roots (Student's *t*-test: WT vs. 4.2, $p=0.016$; WT vs. 6.2, $p=0.008$). However, transgenic lines grown on MS media with 50 mM, 100 mM and 200 mM NaCl all had mean root lengths that were either shorter or the same as wild-type. Consequently, it was concluded that the over-expression of *MecAPX2* in *Arabidopsis* roots did not confer resistance to salt stress.

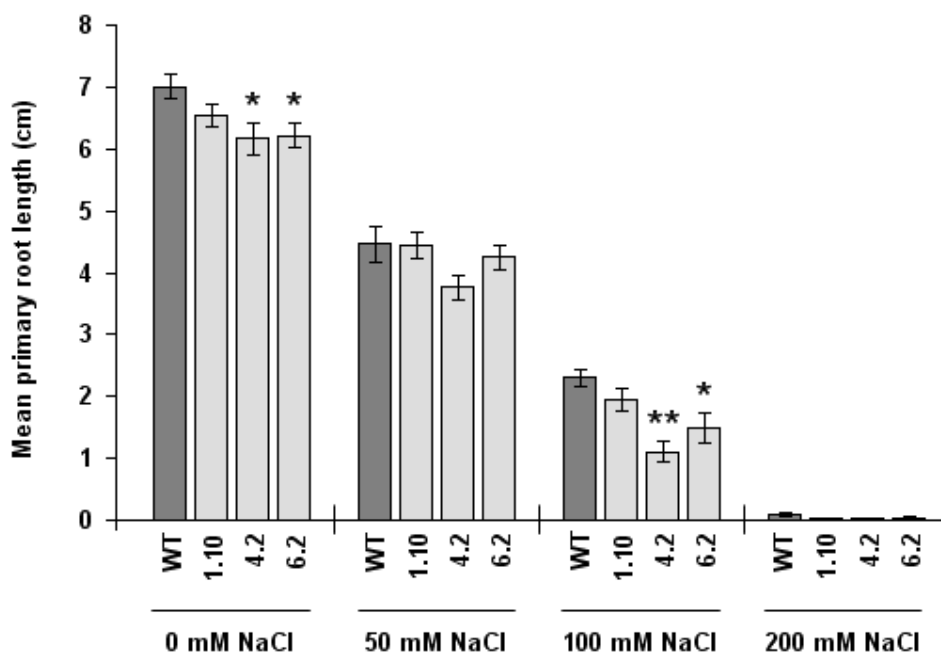


Figure 5.7: Over-expression of *MecAPX2* does not confer resistance to NaCl on *Arabidopsis* roots. Primary root length of wild-type (WT) plants and three transgenic lines (1.10, 4.2 and 6.2) over-expressing *MecAPX2* in a root-specific manner. NaCl concentration provided at the bottom of the x-axis. Asterisks denote root lengths significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, $16 \leq n \leq 20$.

5.2.6 Over-expression of *MecAPX2* in root tissue confers some resistance to H_2O_2 in a soil-based assay

As plant root metabolism can be affected by light exposure (Hemm *et al.*, 2004), an assay using soil-grown plants was carried out to mimic more closely the natural growth environment of roots. Plants were watered equally up to 21 DAG and were then watered daily either with tap water or 0.5 mM H_2O_2 until 29 DAG. Given that the plants were grown in soil, measuring the root length throughout the experiment

was impossible. However, if a plant's roots were more resistant to H_2O_2 , the whole plant should be healthier and therefore grow quicker. As such, the total photosynthetic area was measured at regular intervals as an indicator of the health of the plants (Figure 5.8). Wild-type plants were compared with line 6.2 plants, since line 6.2 demonstrated the most significant increase in APX activity (Figure 5.3) and appeared most resistant to H_2O_2 induced oxidative stress (Figure 5.6). Statistical analysis of the data using the Student's *t*-test revealed there was no difference between any of the samples at any of the time points. This could have been a result of the limitation on the number of plants in each sample. Alternatively, the concentration of H_2O_2 may have been too low to act as an effective stressing agent, especially since the leaf growth rates of wild-type control and wild-type treated plants were not significantly different.

Although no statistical difference was found, the data demonstrated that the growth rate of the photosynthetic tissue of line 6.2 plants watered with H_2O_2 was higher than that of the other plant sets between four and eight days after the treatment began. This indicated that line 6.2 plants had a higher resistance than wild-type plants to H_2O_2 . It also suggested that line 6.2 plants treated with 0.5 mM H_2O_2 were healthier than untreated wild-type and line 6.2 plants.

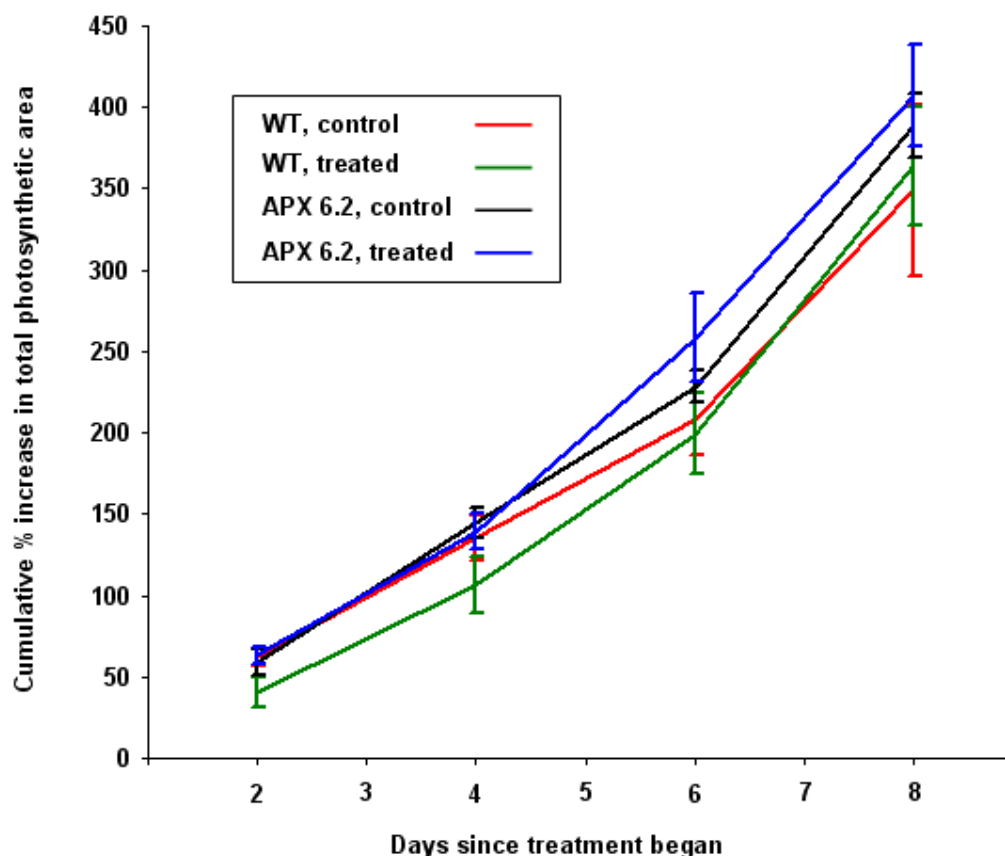


Figure 5.8: Over-expression of *MecAPX2* confers some resistance to H_2O_2 on roots of line 6.2 plants. At 21 DAG, plants were watered daily with either tap water or 0.5 mM H_2O_2 . Total photosynthetic area was measured every two days after commencement of treatment. WT = wild-type, error bars represent \pm SEM, n = 6.

During analysis of photographs it was observed that primary stem elongation had occurred in some of the plants. This hampered the measurement of the photosynthetic area of these plants as stem tissue and cauline leaves physically covered some of the rosette leaf area. Additionally, since elongation of the primary stem is a major developmental event in *Arabidopsis*, the growth rate of the rosette leaves (those being measured) may have been affected. As such, the above experiment was repeated earlier in the life-cycle of the plants, starting at 15 DAG and finishing at approximately 23 DAG and thereby eliminating the problem of primary stem elongation. Furthermore, the concentration of H_2O_2 solution used to water the treated plants was increased to 1.5 mM. This was altered as the 0.5 mM H_2O_2 solution used in the previous experiment did not appear to exert sufficient stress on the wild-type plants to bring about a significantly lower leaf growth rate than untreated wild-type plants.

In the repeated experiment (Figure 5.9), statistical analysis with the Student's *t*-test revealed there was a significant difference between the leaf growth rate of wild-type control and wild-type treated plants at 3 ($p=0.008$), 5 ($p=0.019$) and 8 ($p=0.042$) days after the treatment began. This indicated that the elevated concentration of H_2O_2 used was successfully causing stress. Meanwhile, there was no significant difference between line 6.2 control and treated plants at any time point, suggesting that these plants had some resistance to the stressing agent. Although the data (Figure 5.9) showed that treated line 6.2 plants had a higher leaf growth rate than treated wild-type plants, there was no significant difference at any time point.

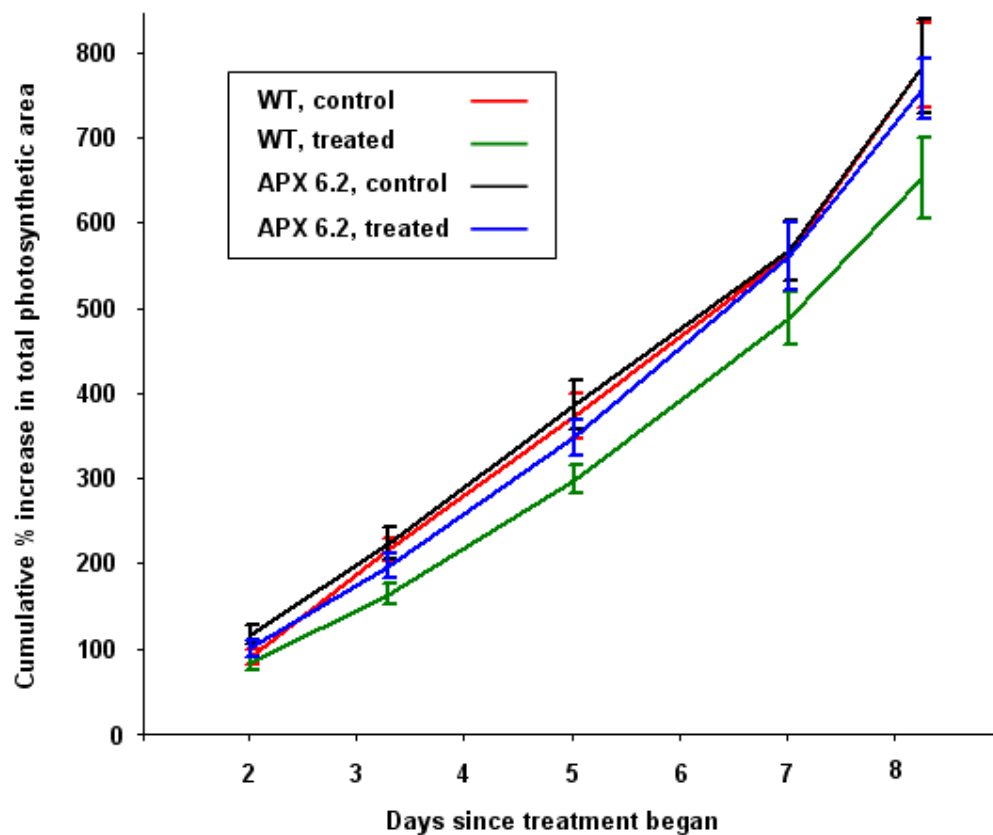


Figure 5.9: Over-expression of *MecAPX2* confers some resistance to H_2O_2 on roots of APX 6.2 *Arabidopsis* plants. At 15 DAG, plants were watered daily with either tap water or 1.5 mM H_2O_2 . Total photosynthetic area was measured at frequent intervals after commencement of treatment. WT = wild-type, error bars represent \pm SEM, $n = 6$.

5.2.7 APX activity increases in wild-type roots in response to stress

The three transgenic *Arabidopsis* lines, 1.10, 4.2 and 6.2, all over-expressed *MecAPX2* in root tissue. Furthermore, line 4.2 showed an increase in total APX transcripts compared to wild-type, and lines 4.2 and 6.2 had significantly higher root APX activity than wild-type. Given this, the level of resistance seen in the above stress assays (Figures 5.4 – 5.9) was lower than expected, especially in response to H₂O₂, the direct substrate of APX. The APX activity of wild-type and transgenic root tissue was measured under normal physiological conditions, but the above experiments compared plants under stress. Therefore, the APX activity of wild-type and transgenic root tissue was assessed under stress conditions to determine if APX activity is modulated as a result. Wild-type and transgenic plants were grown on control MS media and MS media supplemented with 200 µM H₂O₂. After 10 days growth, protein was extracted from root tissue and the APX activity measured.

When H₂O₂ was absent, the transgenic lines all had a higher APX activity than wild-type (Figure 5.10). This was consistent with the previous measurement of APX in unstressed plants (Figure 5.3), although here line 4.2 showed somewhat reduced activity. When the media was supplemented with 200 µM H₂O₂, the root APX level in wild-type and line 4.2 had increased up to the level of lines 1.10 and 6.2, which had remained unchanged. Moreover, a 2.50-fold increase in APX activity was observed in stressed compared to unstressed wild-type roots, indicating APX had been dramatically induced. Statistical analysis of the data was not possible since only one biological replicate was performed.

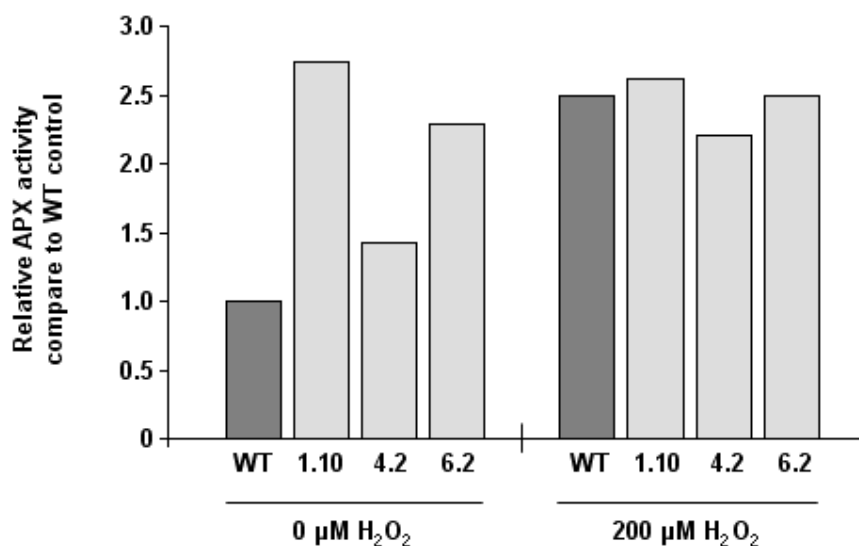


Figure 5.10: APX activity is induced in wild-type *Arabidopsis* roots when H_2O_2 is present in the growing medium. APX activity, in relative HPX equivalents compared to the wild-type control, of wild-type (WT) and transgenic *StPAT::APX2* (1.10, 4.2 and 6.2) *Arabidopsis* root tissue grown on MS media with or without 200 μM H_2O_2 .

5.2.8 Summary (*MecAPX2*)

The *StPAT* promoter drove expression of *MecAPX2* in transgenic *Arabidopsis* root tissue. An increase in total APX transcripts was seen for line 4.2, and all transgenic lines assessed had increased total APX activity in root tissue compared to wild-type. The APX activity in leaf tissue remained at a wild-type level in transgenic lines. Using quantitative stress assays, line 6.2 showed some resistance to oxidative stress generated by H_2O_2 compared to wild-type. However, wild-type root tissue appeared able to induce APX under stress conditions.

5.3 Results – *MecCAT1*

5.3.1 *Arabidopsis* transformation and selection of transgenic lines

Transformation of *Arabidopsis* via floral dip with *A. tumefaciens* harbouring the pDEST™ *MecCAT1* plasmid yielded twelve transgenic lines identified through a hygromycin B screen. gDNA from these twelve lines was PCR genotyped to check the presence of the *StPAT::CAT1* cassette using primer pair 25 (Figure 5.11). All twelve lines were confirmed as transgenic. Differences in product intensities were due to variations in gDNA extraction efficiency.

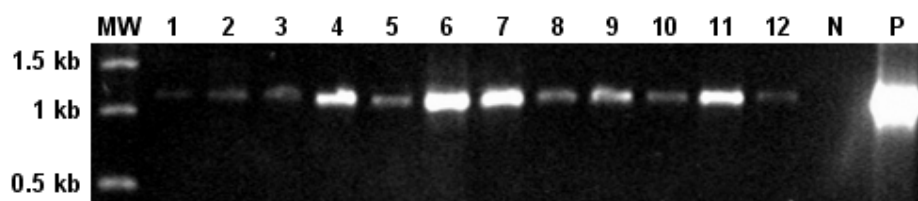


Figure 5.11: Transgenic seedlings identified through a hygromycin B screen are confirmed as harbouring the *StPAT::CAT1* cassette using PCR genotyping. Agarose gel image showing PCR products amplified from gDNA of twelve plants (labelled 1 – 12). MW = molecular weight marker with relevant sizes to the left of the gel image. N = negative control (wild-type gDNA), P = positive control (pDEST™ *MecCAT1* plasmid DNA as template). Expected product size is 1,141 bp.

Of the twelve transgenic lines (T_1 generation), seven were identified as carrying a single-insertion (T_2). Subsequently, homozygous lines (T_3) were found for six of the seven single-insertion lines. Three of these lines were randomly chosen and carried forward for use in the subsequent experiments. These lines were referred to as CAT 2.3, CAT 3.8 and CAT 9.2 and the transgenic cassette referred to as *StPAT::CAT1*.

5.3.2 *Arabidopsis* harbouring the *StPAT::CAT1* cassette over-express the transgene in root tissue

The expression level of *MecCAT1* in lines 2.3, 3.8 and 9.2 was assessed by sqPCR (Figure 5.12). Lines 2.3 and 9.2 were expressing highly at a similar level, while 3.8 showed very low transgene expression. The total *CAT* expression was

affected accordingly, with lines 2.3 and 9.2 substantially higher than wild-type and 3.8. Interestingly, the total *APX* expression of the three transgenic lines seemed elevated above the wild-type level, which suggested that an increase in *CAT* expression could modulate *APX* expression. The total level of *SOD* transcripts was unaffected by the increase in *CAT* expression.

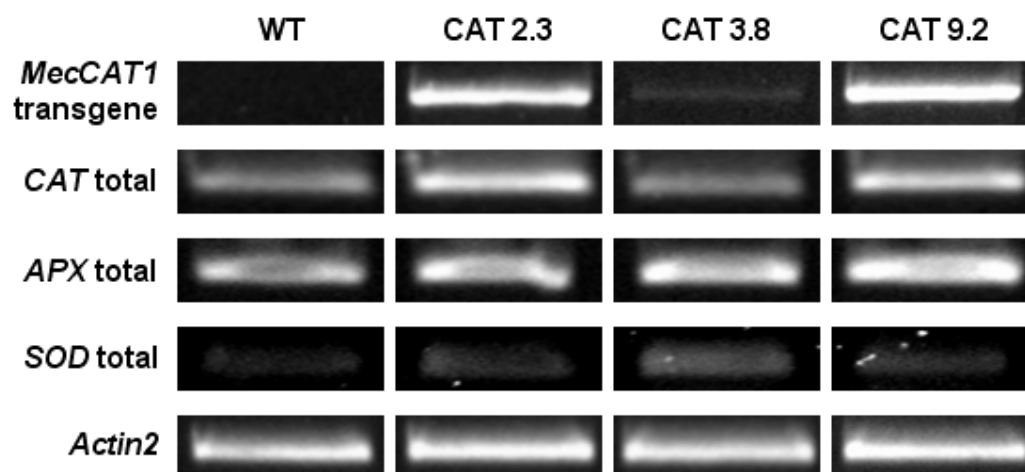


Figure 5.12: Transgenic *Arabidopsis* lines harbouring the *StPAT::CAT1* cassette show varying levels of transgene expression in root tissue. Number of amplification cycles: *MecCAT1* – 23; *CAT* total – 23; *APX* total – 23; *SOD* total – 23; *Actin2* – 21. Primer pairs: *MecCAT1* – 26; *CAT* total – 22; *APX* total – 21; *SOD* total – 23; *Actin2* – 24. WT = wild-type, Col-0.

5.3.3 An increase in *MecCAT1* mRNA does not result in a significant increase in *CAT* activity

The transcript level of gene families does not always correlate with the amount of active protein. As such, the activity of *CAT* in the transgenic root samples was measured to establish if it too had increased from the wild-type level.

The wild-type activity of *CAT* was significantly higher in leaf tissue than in root tissue (Student's *t*-test: WT leaf vs. WT root, $p=0.009$), while the leaf *CAT* activities of transgenic lines over-expressing *MecCAT1* were not significantly different from wild-type (Student's *t*-test: WT vs. 2.3, $p=0.404$; WT vs. 3.8, $p=0.325$; WT vs. 9.2, $p=0.337$) (Figure 5.13).

Further statistical analysis demonstrated a lack of significant difference between the *CAT* activity of the transgenic and wild-type root samples (Student's *t*-

test: WT vs. 2.3, $p=0.110$; WT vs. 3.8, $p=0.566$; WT vs. 9.2, $p=0.106$). A shortage of biological replicates may have affected the statistical analysis. The root CAT enzyme activities of the four lines tested here are consistent with the results from the total CAT mRNA levels (Figure 5.12), with 2.3 and 9.2 higher than 3.8 and wild-type. Furthermore, the increase in CAT activity was restricted to root tissue, reinforcing the supposition that the *StPAT* promoter is driving root-specific expression. The CAT activity in leaf tissue decreased, although not significantly.

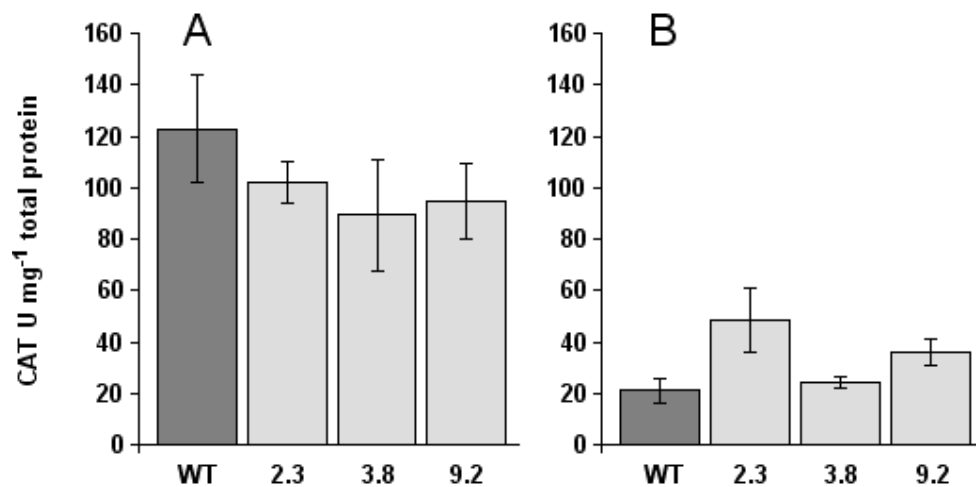


Figure 5.13: Transgenic *Arabidopsis* lines harbouring the *StPAT::CAT1* cassette do not have significantly increased CAT activity in root tissue. Mean CAT activity is given for *Arabidopsis* leaf (A) and root (B) tissue. 2.3, 3.8 and 9.2 refer to independent transformation lines harbouring *StPAT::CAT1*, WT = wild-type, Col-0. Asterisks denote enzyme activities significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, $n = 3$.

The remaining three independent transgenic lines were also assessed for root CAT activity. None of the lines showed a significant increase compared to wild-type root samples (data not shown).

5.3.4 Over-expression of *MecCAT1* in *Arabidopsis* root tissue does not confer abiotic stress resistance

Given that the CAT activity of the transgenic lines had not increased significantly, it was unlikely that the stress resistance of these lines would be significantly modulated. A small-scale screen was performed to establish if any of the transgenic lines showed resistance to a range of stressing agents.

When grown under the varying stresses, the transgenic CAT lines did not show any observable resistance (Figure 5.14). Under normal physiological conditions (Figure 5.14A), lines 3.8 and 9.2 were slightly larger than wild-type with a more developed root system.

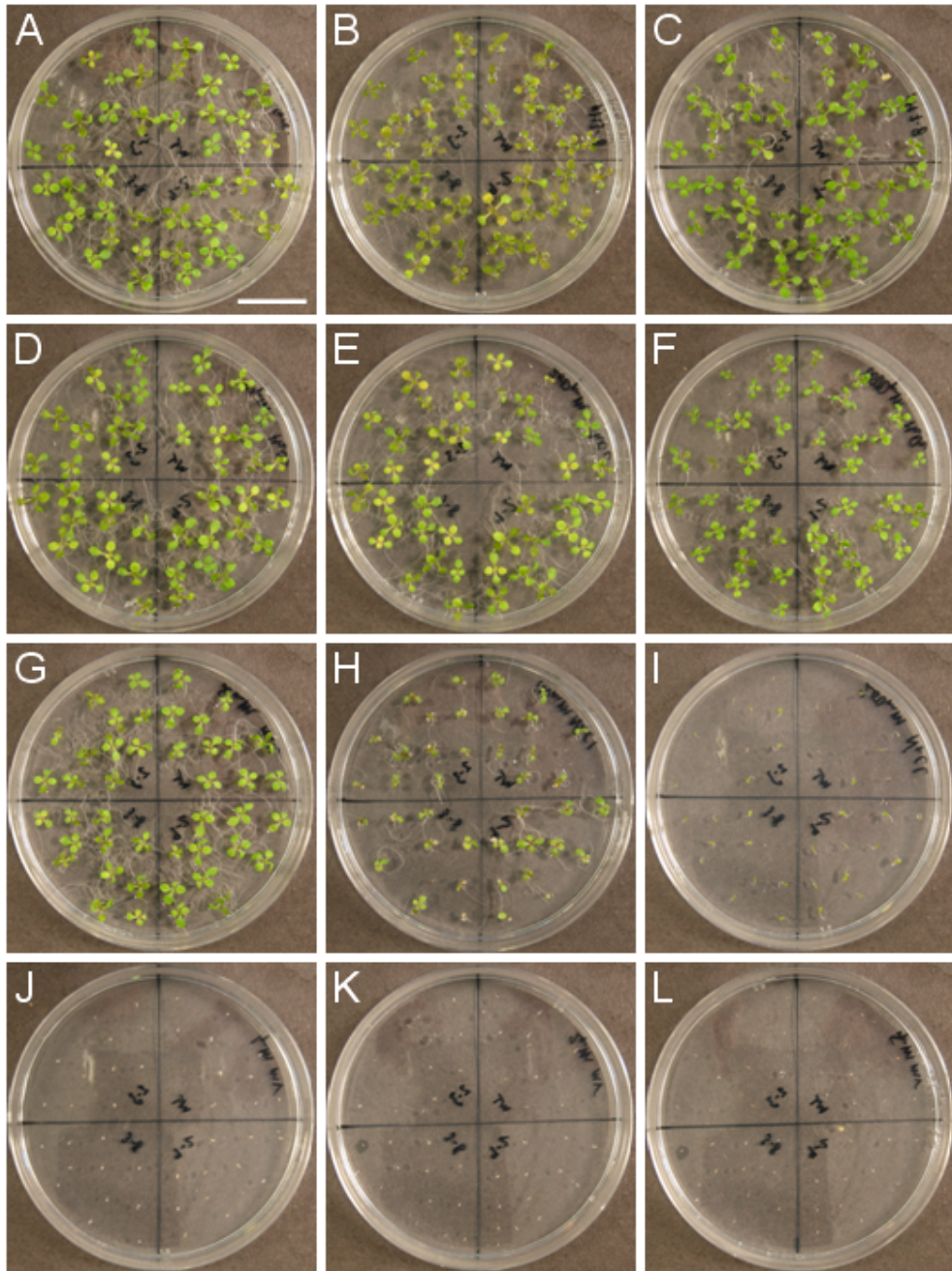


Figure 5.14: Over-expression of *MecCAT1* in *Arabidopsis* roots does not confer resistance to abiotic stress. On each Petri dish: wild-type (top right); CAT 2.3 (top left); CAT 3.8 (bottom left); and CAT 9.2 (bottom right). Seeds were germinated and grown on MS media containing stress chemicals: (A) control; (B) media at pH 3.8; (C) media at pH 7.8; (D) 40 μM H_2O_2 ; (E) 200 μM H_2O_2 ; (F) 1,000 μM H_2O_2 ; (G) 50 mM NaCl; (H) 100 mM NaCl; (I) 200 mM NaCl; (J) 1 μM MV; (K) 2 μM MV; and (L) 5 μM MV. Bar represents 2 cm, all photographs taken at the same magnification.

5.3.5 Summary (*MecCAT1*)

Arabidopsis plants were transformed with the *StPAT::CAT1* cassette. Two lines showed strong expression of the transgene in root tissue. The CAT activity of root tissue in these lines increased, although this was not significant. In a small-scale screen, the lines with increased root CAT activity were not more resistant to oxidative stress. Given this result, and the insignificant increase in root CAT activity in transgenic lines, further investigation of these lines was not carried out.

5.4 Results – *MecSOD2*

5.4.1 *Arabidopsis* transformation and selection of transgenic lines

Arabidopsis plants were transformed with the pDEST™ *MecSOD2* plasmid. A hygromycin B screen identified seventeen positive lines. gDNA from these lines was genotyped via PCR to confirm the presence of the expression cassette using primer pair 27 (Figure 5.15). All lines were confirmed as transgenic.

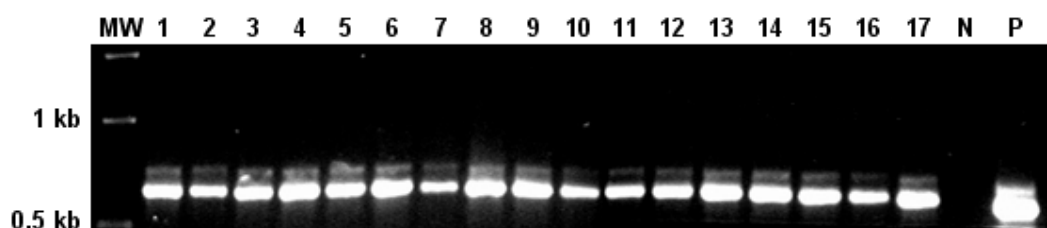


Figure 5.15: Transgenic seedlings identified through a hygromycin B screen are confirmed as harbouring the *StPAT::SOD2* cassette using PCR genotyping. Agarose gel image showing PCR products amplified from gDNA of seventeen plants (labelled 1 – 17). MW = molecular weight marker with relevant sizes to the left of the gel image. N = negative control (wild-type gDNA), P = positive control (pDEST™ *MecSOD2* plasmid DNA as template). Expected product size is 661 bp.

Of the seventeen transgenic lines (T_1), eight carried a single-insertion (T_2). Homozygous lines (T_3) were identified for six of the eight single-insertion lines. Three lines were chosen at random for the subsequent experiments and were referred to as SOD 4.9, SOD 5.8 and SOD 7.8. The expression cassette was referred to as *StPAT::SOD2*.

5.4.2 *Arabidopsis* harbouring the *StPAT::SOD2* cassette over-express the transgene in root tissue

The expression level of *MecSOD2* in transgenic and wild-type roots was measured to establish if the *StPAT* promoter was driving over-expression of the transgene in this tissue. A range of *MecSOD2* expression levels was observed for the three transgenic lines, with line 4.9 expressing most highly and line 7.8 expressing the least, while no transgene expression was seen in wild-type roots (Figure 5.16). The over-expression of the transgene affected the total *SOD* transcript level. Line

4.9 showed an obvious increase in total *SOD* expression. Surprisingly, given that line 7.8 was expressing the transgene at a low level, the total *SOD* expression of this line was also high, at a similar level to line 4.9. The total *APX* and *CAT* expression levels remained unchanged across the wild-type and transgenic lines.

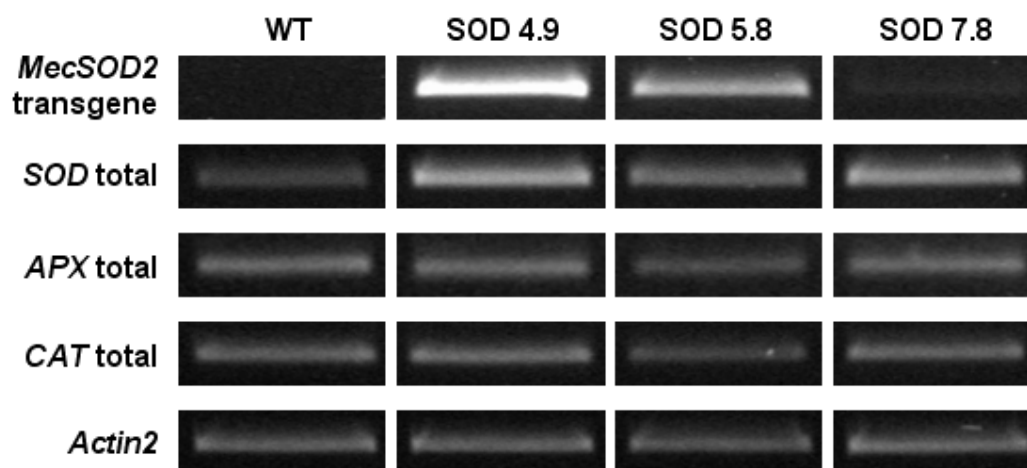


Figure 5.16: Transgenic *Arabidopsis* lines harbouring the *StPAT::SOD2* cassette show varying levels of transgene expression in root tissue. Number of amplification cycles: *MecSOD2* – 29; *SOD* total – 32; *APX* total – 23; *CAT* total – 25; *Actin2* – 22. Primer pairs: *MecSOD2* – 28; *SOD* total – 23; *APX* total – 21; *CAT* total – 22; *Actin2* – 24. WT = wild-type.

5.4.3 An increase in *MecSOD2* mRNA results in a significant increase in SOD activity

The total SOD activity of transgenic and wild-type root tissue was measured to establish if the observed over-expression of *MecSOD2* had modulated the total activity of the enzyme. The SOD activity of leaf tissue was also assessed to confirm the tissue-specificity of the promoter.

There was no significant difference in the wild-type SOD activity between leaf and root tissue (Student's *t*-test: WT leaf vs. WT root, $p=0.680$) (Figure 5.17). The leaf SOD activity was variable in the transgenic lines, although none showed a significant difference from wild-type (Student's *t*-test: WT vs. 4.9, $p=0.545$; WT vs. 5.8, $p=0.184$; WT vs. 7.8, $p=0.160$). Importantly, the root SOD activities of the transgenic lines had all increased from the wild-type level, with 4.9 and 7.8 significantly higher (Student's *t*-test: WT vs. 4.9, $p=0.005$; WT vs. 5.8, $p=0.115$;

WT vs. 7.8, $p=0.050$). This strengthened the view that *StPAT* was causing over-expression of *MecSOD2* in a root-specific manner.

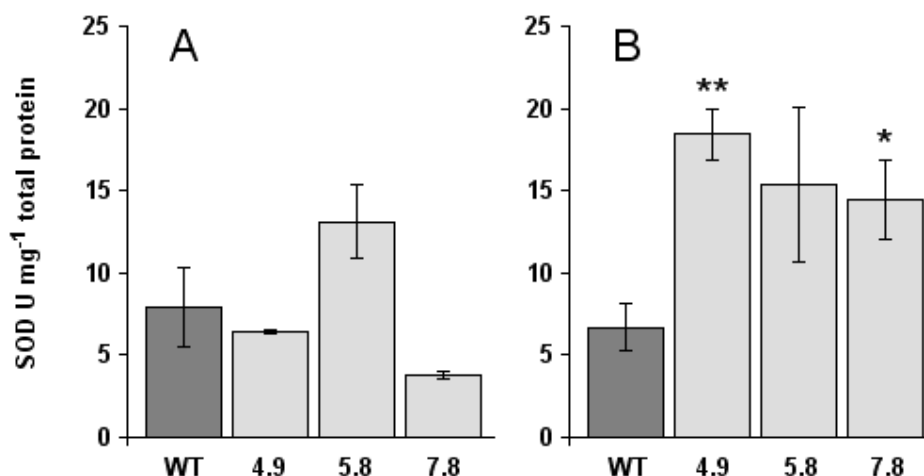


Figure 5.17: Transgenic *Arabidopsis* lines harbouring the *StPAT::SOD2* cassette have increased SOD activity in root tissue. Mean SOD activity is given for *Arabidopsis* leaf (A) and root (B) tissue. 4.9, 5.8 and 7.8 refer to independent transformation lines harbouring *StPAT::SOD2*, WT = wild-type, Col-0. Asterisks denote enzyme activities significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, n = 3.

5.4.4 An increase in root SOD activity does not confer resistance to oxidative stress

The three transgenic lines carrying the *StPAT::SOD2* cassette had a higher root SOD activity than wild-type plants. It was expected that this would confer some resistance to the effects of oxidative stress since SOD directly detoxifies superoxide radicals, which are essential for root growth and root hair development but during stress accumulate to high levels and cause oxidative damage to cells (Dunand *et al.*, 2007). As such, a small-scale assay was employed to screen the transgenic plants for oxidative stress resistance.

Under normal physiological conditions, the transgenic lines are similar in appearance to wild-type after 7 days growth (Figure 5.18A). Under stress conditions (Figure 5.18B – 5.18L), over-expression of *MecSOD2* in root tissue did not appear to confer an advantage on the transgenic lines when compared to wild-type.

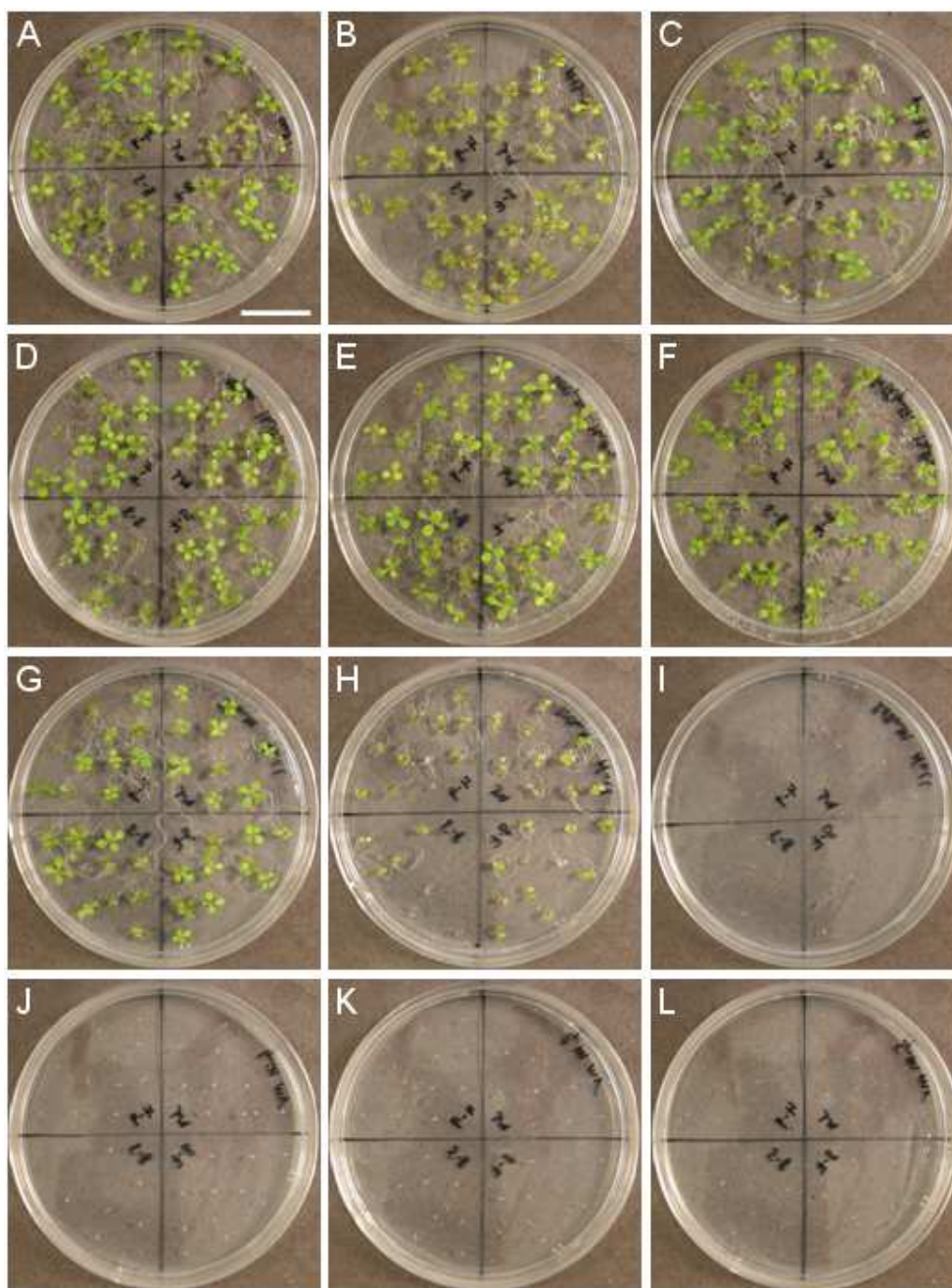


Figure 5.18: Over-expression of *MecSOD2* in *Arabidopsis* roots does not confer resistance to abiotic stress. On each Petri dish: wild-type (top right); SOD 4.9 (top left); SOD 5.8 (bottom left); and SOD 7.8 (bottom right). Seeds were germinated and grown on MS media containing stress chemicals: (A) control; (B) media at pH 3.8; (C) media at pH 7.8; (D) 40 μM H_2O_2 ; (E) 200 μM H_2O_2 ; (F) 1,000 μM H_2O_2 ; (G) 50 mM NaCl; (H) 100 mM NaCl; (I) 200 mM NaCl; (J) 1 μM MV; (K) 2 μM MV; and (L) 5 μM MV. Bar represents 2 cm, all photographs taken at the same magnification.

The transgenic lines were not expected to out-perform wild-type plants when grown on MS media supplemented with H₂O₂ since SOD is not able to detoxify H₂O₂. Surprisingly, they did not appear able to out-perform wild-type plants when grown on MV either. This herbicide generates superoxide radicals, the direct substrate of SOD.

Since the small-scale assay used above was qualitative, an assay that could quantify the degree of resistance was used to screen the transgenic *StPAT::SOD2* plants more rigorously.

5.4.5 Root-specific over-expression of *MecSOD2* in *Arabidopsis* roots confers some resistance to low concentrations of MV

Transgenic and wild-type plants were grown on MS media plates supplemented with MV and incubated 5° from vertical. After 8 days growth, photographs were taken and the primary root lengths were measured.

The control plates demonstrated that under normal physiological conditions, the growth of transgenic lines was not significantly different from that of wild-type plants (Figure 5.19). When the plates were supplemented with 100 nM MV, roots of lines 5.8 and 7.8 were significantly longer than wild-type (Student's *t*-test: WT vs. 5.8, *p*=0.043; WT vs. 7.8, *p*=0.003) indicating that over-expression of *MecSOD2* was conferring some stress resistance in these lines. Interestingly, lines 5.8 and 7.8 were expressing *MecSOD2* at a lower level (Figure 5.16) and had a lower root SOD activity (Figure 5.17) than line 4.9 but were more resistant to the stress induced by MV. At both 250 nM and 500 nM MV, the mean root length of transgenic plants was not significantly different from wild-type.

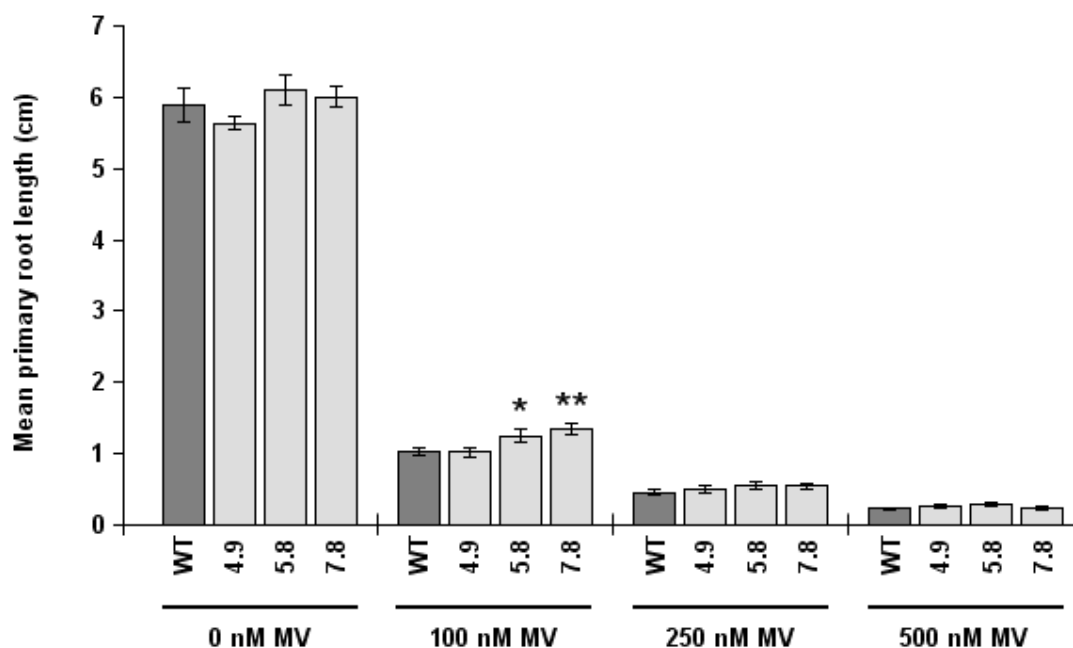


Figure 5.19: Over-expression of *MecSOD2* confers some resistance to low MV concentrations on *Arabidopsis* roots. Primary root length of wild-type (WT) plants and three transgenic lines (4.9, 5.8 and 7.8) over-expressing *MecSOD2* in a root-specific manner. MV concentration provided at the bottom of the x-axis. Asterisks denote root lengths significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, $14 \leq n \leq 20$.

The above experiment was repeated, supplementing the MS media with NaCl in place of MV (Figure 5.20). On control plates here, the pattern of mean root lengths across lines was identical to the pattern seen on the control plates in the H_2O_2 experiment (Figure 5.19). This indicated that this method of assessing plant health was reliable. At all concentrations of NaCl, the mean root lengths of transgenic lines were not significantly different from wild-type.

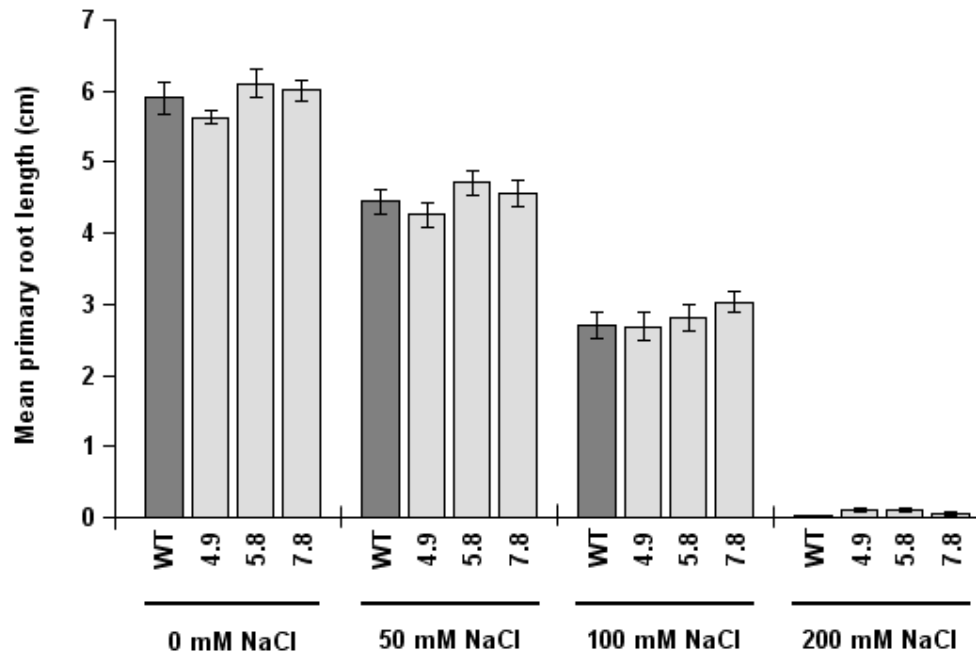


Figure 5.20: Over-expression of *MecSOD2* does not confer resistance to NaCl on *Arabidopsis* roots. Primary root length of wild-type (WT) plants and three transgenic lines (4.9, 5.8 and 7.8) over-expressing *MecSOD2* in a root-specific manner. NaCl concentration provided at the bottom of the x-axis. Asterisks denote root lengths significantly different from wild-type at the 95% (*) and 99.5% (**) level using a Student's *t*-test. Error bars represent \pm SEM, $14 \leq n \leq 20$.

5.4.6 Summary (*MecSOD2*)

Of three randomly chosen *Arabidopsis* lines containing the *StPAT::SOD2* cassette, line 4.9 had the highest level of transgene expression and the highest root SOD activity. However, of the three transgenic lines, 4.9 was the least resistant to the superoxide-generating herbicide MV. Lines 5.8 and 7.8 were more resistant than wild-type to MV present at a low concentration. No resistance to any other source of oxidative stress was observed.

5.5 Results – Life history traits of transgenic lines

Analysis of the expression pattern conferred by the *StPAT* promoter in *Arabidopsis* revealed that the promoter was driving expression chiefly in the root tissue. However, *StPAT*-derived expression was also observed in mature reproductive organs and at leaf hydathodes (Figure 4.2). It was therefore necessary to establish if transgene expression at these locations modulated the development of transgenic plants in any way. Five phenotypes were scored in transgenic lines and compared to wild-type (Table 5.1). Each individual line was assessed independently, with data then averaged for each set of transgenic lines.

It should be noted first that the *GusP* reporter lines were not significantly different from wild-type for any of the five phenotypes. Germination efficiency was the same for transgenic lines as for wild-type. This was expected since no reporter gene activity driven by the *StPAT* promoter was observed until after germination had occurred. The mean root length of *CAT1* lines was significantly higher than all other lines tested (Student's *t*-test: WT vs. *CAT1*, $p=0.009$; *APX2* vs. *CAT1*, $p=0.008$; *SOD2* vs. *CAT1*, $p=0.028$; *GusP* vs. *CAT1*, $p=0.020$). Given that the root CAT activity had increased in the three transgenic lines (Figure 5.13), it is likely that the elevated level of CAT is conferring an advantage to *Arabidopsis* roots under normal physiological conditions. It is unclear why an increased CAT activity should confer such an advantage, and not increased APX and SOD activities. The mean flowering time of lines harbouring the *StPAT::CAT1* cassette was significantly earlier than both wild-type and the *GusPlus* reporter lines, occurring on average over a day earlier than wild-type (Mann-Whitney *U*-test: WT vs. *CAT1*, $p=0.0091$; *GusP* vs. *CAT1*, $p=0.0483$). It is not known whether this is due to the modulation of CAT activity, since this was not assessed in floral tissue. However, the fertility of *CAT1* lines was also significantly reduced compared to wild-type (Mann-Whitney *U*-test: WT vs. *CAT1*, $p=0.0098$). It is likely that expression of *MecCAT1* in mature reproductive organs, an artefact of *StPAT* promoter activity, is implicated in this phenotype alteration. Interestingly, the fertility of *APX2* and *SOD2* lines had also decreased, although not significantly. The mean seed number per pod was significantly reduced in *APX2* and *SOD2* lines compared to wild-type (Mann-Whitney *U*-test: WT vs. *APX2*, $p=0.0318$; WT vs. *SOD2*, $p=0.0234$). This could also be an effect of transgene expression in mature reproductive organs driven by the *StPAT* promoter.

Although the *CAT1* plants demonstrated the smallest increase in root enzyme activity of the transgenic lines, they exhibited the most alteration in the phenotypes assessed here.

Table 5.1: Phenotype analysis of transgenic and wild-type plants.

Phenotype	Wild-type	<i>APX2</i>	<i>CAT1</i>	<i>SOD2</i>	<i>GusP</i>
Germination (%)	98.03 ± 0.50 ^a	98.17 ± 0.91 ^a	98.52 ± 0.31 ^a	98.50 ± 0.66 ^a	98.16 ± 0.59 ^a
Primary root length (cm)	3.33 ± 0.08 ^a	3.28 ± 0.10 ^a	3.66 ± 0.10 ^b	3.35 ± 0.10 ^a	3.37 ± 0.08 ^a
Flowering time (DAG)	31.67 ± 0.35 ^a	30.67 ± 0.40 ^{ab}	30.33 ± 0.44 ^b	30.67 ± 0.27 ^{ab}	31.07 ± 0.30 ^a
Fertility (%)	98.85 ± 0.51 ^a	95.56 ± 2.41 ^{ab}	93.48 ± 2.05 ^b	94.42 ± 2.77 ^{ab}	98.10 ± 0.53 ^{ab}
Seeds per pod	48.33 ± 1.01 ^a	44.93 ± 1.33 ^b	45.60 ± 1.19 ^{ab}	44.47 ± 1.21 ^b	48.13 ± 1.01 ^a

Values given are means ± SEM. Superscript letters denote significant differences at the 95% level using the Student's *t*-test (germination, primary root length) or Mann-Whitney *U*-test (flowering time, fertility, number of seeds per pod).

Lines used for each set were: *APX2* – 1.10, 4.2, 6.2; *CAT1* – 2.3, 3.8, 9.2; *SOD2* – 4.9, 5.8, 7.8; *GusP* – 2.8, 7.1 and 16.5.

5.6 Discussion

5.6.1 Increasing the root antioxidant enzyme activity of *Arabidopsis* plants

Four of the nine transgenic *Arabidopsis* lines generated showed a significant increase in enzyme activity in root tissue compared to wild-type. Additionally, the activity of the enzymes in leaf tissue remained at wild-type levels. It was assumed therefore that the *StPAT* promoter was driving transgene expression in a root-specific manner, in agreement with the results seen with *GusP* reporter lines.

Two of the three transgenic *Arabidopsis* lines over-expressing *MecAPX2* demonstrated a significant increase in APX activity compared to wild-type. However, while line 4.2 showed the highest level of transgene expression among the three lines, it had the lowest mean enzyme activity. This disparity between expression and activity could have been an effect of the assays used, since 'MecAPX2 transgene' and 'total APX' transcripts were assessed using sqPCR, whereas the enzyme assay measured both APX and GuPX activity. The lack of correlation may also have arisen from the specificity of the primers used in the sqPCR step. Degenerate primers were designed to amplify the 'total APX' transcripts, as shown in the sqPCR gel image. However, it was not possible to design one primer pair to amplify the one transgenic cassava cDNA and all the native *Arabidopsis* cDNAs. As a result, the degenerate primers were designed to amplify the most sequences possible. Consequently not all transcripts were included in the 'total APX' bands in the sqPCR, whereas the enzyme activity assay quantified the total activity.

An alternative explanation for the lack of correlation between APX mRNA and APX activity is post-transcriptional regulation. Transcript levels of *cAPX* in spinach leaves accumulated in response to stress, while the protein activity increased only to a lesser extent (Yoshimura *et al.*, 2000). Independent research had previously demonstrated that while *cAPX* transcript levels accumulated during recovery from drought, only a small fraction was associated with polysomes and therefore involved in synthesising *cAPX* protein (Mittler & Zilinskas, 1994). Additionally, virus-induced programmed cell death in tobacco led to the accumulation of *cAPX* transcripts, although polypeptide elongation during translation did not take place and so the level of APX protein declined (Mittler *et al.*, 1998). Ultimately, the conflict between expression level and enzyme activity is unimportant as two lines with significantly higher APX activities than wild-type were isolated.

The CAT activity of leaf tissue was significantly higher than the activity in root tissue in wild-type plants. This agreed with existing data (Frugoli *et al.*, 1996) and strongly supported a role for CAT in scavenging H₂O₂ generated by the photosynthetic machinery (Yang & Poovaiah, 2002). Transgenic lines over-expressing *MecCAT1* showed a correlation between transgene expression and total CAT activity in root tissue. However, the increase in enzyme activity from the wild-type level was not statistically significant. The regulation of *CAT* expression is complex and is affected by both environmental and developmental signals, including the circadian clock, light, oxidative stress and pathogen invasion (McClung, 1997). One or several of these signals may induce *CAT* expression through a mitogen-activated protein kinase (MAPK) cascade, initiated by ABA (Xing *et al.*, 2008). Evidence also exists that suggests *CAT* expression may be regulated at the post-transcriptional level in cotton, a phenomenon that partially depends on exposure to light (Ni & Trelease, 1991). CAT activity in mammalian cell lines is controlled at the post-translational level. High levels of H₂O₂ induce the binding of two tyrosine kinases to form a heterodimer, which is then able to bind to and phosphorylate CAT at two tyrosine residues, thereby enhancing the activity of the enzyme (Cao *et al.*, 2003). A similar system has been uncovered in plants – calcium and calmodulin were shown to act together, binding to and thereby increasing the catalytic activity of a tobacco CAT (Yang & Poovaiah, 2002). Under normal physiological conditions, low concentrations of H₂O₂ may act to maintain CAT in an inactive form. Thus the insignificant increase in CAT activity in the transgenic lines generated here compared to wild-type may be a consequence of post-translational regulation of wild-type and transgenic CAT protein. Alternatively, the level of transgene expression seen here may simply be insufficient to significantly increase the root CAT activity of *Arabidopsis* root tissue.

The relative levels of *SOD* transcripts in lines over-expressing *MecSOD2* corresponded approximately to the activity of SOD in root tissue. This indicated a straightforward relationship between mRNA and protein levels. Lines 4.9 and 7.8 possessed significantly higher root SOD activities than wild-type plants.

5.6.2 Enzyme activity vs. oxidative stress resistance

Given that four of the transgenic lines produced here showed significantly increased antioxidant activity in root tissue, the level of resistance to oxidative stress shown by these lines is somewhat disappointing.

The transgenic plants over-expressing *MecAPX2* showed no resistance to the majority of stresses imposed on them, only out-performing wild-type plants when H_2O_2 was present at a high concentration. Other similar studies involving the production of transgenic plants over-expressing *APX* genes have predominantly shown significant gains in stress tolerance after comparable increases in enzyme activity. Transgenic tobacco plants over-expressing pepper *ASCORBATE PEROXIDASE-LIKE 1* (*CaPOA1*) had *APX* activities up to two-fold that of wild-type plants (Sarowar *et al.*, 2005). Stress assays entailing the application of MV to leaf tissue demonstrated a considerable reduction of leaf bleaching in response to the herbicide in transgenic lines. Another study generated *Arabidopsis* plants with *APX* activity between two- and six-fold higher than wild-type plants, and again the transgenic lines showed an unambiguous reduction in bleaching (Murgia *et al.*, 2004). The method of screening for stress resistance may therefore be important, since these investigations employed different assay procedures to those used here. However, a leaf-based assay was not suitable for this investigation as transgene expression was predominantly limited to root tissue. Consequently, plants were grown *in vitro* on stressing chemicals and the primary root length measured, thereby establishing a quantitative assay. This method had been used previously to successfully discriminate between stress resistant and susceptible plants, although wild-type plants were compared with lines with reduced expression of *APX* genes (Miller *et al.*, 2007). The lines generated in this study all possessed *APX* activities at least three-fold that of wild-type plants, but did not show resistance to NaCl, pH alterations, drought, MV or H_2O_2 at most concentrations using *in vitro* assays.

Further data presented here subsequently suggested that the lack of oxidative stress resistance of lines over-expressing *MecAPX2* may not have been a consequence of the assay procedure. When grown on MS media with no additions, transgenic lines possessed up to 2.73-fold the root *APX* activity of wild-type plants. However, when plants were grown on MS media supplemented with 200 μM H_2O_2 , the root *APX* activity of wild-type plants had increased significantly, and was now at a similar level to the transgenic plants. The cause of the induction

of APX activity in H₂O₂-stressed plants is unclear. There is evidence to suggest that expression of an *Arabidopsis* zinc finger gene (*ZAT12*) is up-regulated by H₂O₂ treatment, and that *ZAT12* together with an unknown signal, is essential for the expression of cytosolic *APX1* under oxidative stress conditions (Desikan *et al.*, 2001; Rizhsky *et al.*, 2004). It is possible that this signalling pathway is activated in the wild-type plants under stress in these assays, while transgenic plants carrying the cassava-derived transgene under the control of the potato-derived *StPAT* promoter are not subject to this regulation. As such, the advantage conferred on the transgenic plants from *MecAPX2* over-expression would be lost under H₂O₂-induced stress due to a wild-type-specific increase in *APX* expression. *ZAT12* up-regulation also occurred in response to other stress treatments, including wounding, drought, high and low temperature and MV (Davletova *et al.*, 2005; Hruz *et al.*, 2008), although the generation of H₂O₂ common to these stresses may be the underlying cause of *ZAT12* up-regulation.

The root CAT activity in lines over-expressing *MecCAT1* was not significantly higher than wild-type (between 1.15- and 2.38-fold). As a result, transgenic lines over-expressing *MecCAT1* showed a similar level of stress resistance to wild-type plants. Until lines with significantly higher root CAT activities are isolated, stress resistance assays are somewhat meaningless since the cause of any resistance seen cannot be convincingly reasoned to be due to transgene expression.

Two of the three lines over-expressing *MecSOD2* possessed root SOD activities that were significantly higher than the wild-type level. However, these lines showed no resistance to the majority of stresses applied, with tolerance only to MV at 100 nM. Interestingly, the line with the highest mean root SOD activity was the only line to not demonstrate a significant resistance to MV at this or any other concentration. The reason for an absence of stress resistance in these lines is likely to be due to post-transcriptional regulation of the wild-type *Arabidopsis* SOD genes by micro RNA. Micro RNAs are 20-24 base pair, genome-encoded, single-stranded RNAs, each of which is able to bind to one or a few mRNA transcript targets in a sequence-specific manner (Sunkar & Zhu, 2004). When plants are unstressed, micro RNA 398 (miR398) binds to *Arabidopsis* *Cu/ZnSOD1* and *Cu/ZnSOD2* mRNA with extensive sequence complementarity, thereby initiating mRNA degradation via the RNA-induced silencing complex (RISC)

(Sunkar *et al.*, 2006). This mechanism maintains Cu/ZnSOD at a low level when oxidative stress is minimal, and also helps determine the tissue- and development-specific expression pattern of *Cu/ZnSOD1* and *Cu/ZnSOD2*. During diverse sources of oxidative stresses, transcription of miR398 is down-regulated, allowing transcripts of the two *Cu/ZnSOD* genes to accumulate, ultimately resulting in more ROS-scavenging Cu/ZnSOD enzyme (Sunkar *et al.*, 2006; Sunkar *et al.*, 2007; Jagadeeswaran *et al.*, 2009). This potential regulatory mechanism of two *Arabidopsis* *Cu/ZnSOD* genes bears resemblance to the regulation of *APX1* via H₂O₂-induced *ZAT12* since it is likely that only native transcripts are affected by this mechanism; miR398 was complementary to *Arabidopsis* *Cu/ZnSOD2* at sixteen of twenty-one positions, but complementary to *MecSOD2* at only twelve of twenty-one positions. Therefore, under stress conditions SOD activity in wild-type plants might have been significantly augmented in the assays performed in this study. It is likely that *APX* and *CAT* transcripts are similarly affected by miRNA regulation. However, miRNA research is in its infancy and to date miRNAs that regulate *APX* and *CAT* in *Arabidopsis* have not been identified.

It appears then that the lack of oxidative stress resistance shown by the transgenic lines generated in this study may be largely dependent on the induction of antioxidant enzyme activity in wild-type *Arabidopsis* plant roots under stress conditions. In cassava storage roots, the induction of antioxidant enzyme activity in untransformed plants is either absent or insufficient in timing and/or magnitude to confer tolerance to the ROS-mediated process of PPD. As such, over-expression of the antioxidant genes in cassava storage roots may still be a viable strategy for increasing their shelf life.

5.6.3 Further Work

To analyse further the oxidative stress resistance of line APX 6.2, the soil-based experiment could be modified further, using a higher concentration of H₂O₂ solution to water the plants. The H₂O₂ in the watering solution appeared susceptible to degradation after application to the soil; even very high concentrations (up to 1.5 mM) were unable to consistently reduce the growth of wild-type treated plants by a significant amount compared to wild-type control plants. Under more intense stress conditions, line 6.2 plants are expected to out-perform wild-type to a higher degree than that previously demonstrated (Figure

5.9). The experiment assessing APX activities of roots grown on MS media with and without H₂O₂ (Figure 5.10) should also be repeated to generate data that can be analysed statistically to establish if the responses seen were real. This experiment should also be performed using the SOD lines, replacing the H₂O₂ with MV, to establish if post-transcriptional miRNA-regulation of wild-type SOD transcripts is occurring. All future assessments of APX activity should use an assay that will detect APX and not GuPX, such as that based on the differing sensitivities of the two enzymes to *p*-chloromercuribenzoate (Amako *et al.*, 1994). Further screening of transgenic *Arabidopsis* lines harbouring the *StPAT::CAT1* cassette should be carried out in order to identify lines with a significantly higher root CAT activity than wild-type.

Production and assessment of transgenic lines with knocked-down expression of APX, CAT and SOD transcripts in root tissue would provide an indication of the relative importance of each of these enzymes during oxidative stress in *Arabidopsis* roots. Transgenic lines carrying antisense cassava gene sequences driven by the *StPAT* promoter have been produced but not analysed. The degree to which the native *Arabidopsis* genes would be down-regulated, given that the antisense gene sequence is from cassava, is unlikely to be high. However, only partial regions of the cassava gene sequences were used to build the antisense expression plasmids, with regions showing high homology to equivalent *Arabidopsis* gene sequences preferentially chosen.

It is known that sources of oxidative stress induce large suites of genes, and that these groups consist of different genes depending on the type of stress experienced (Mittler *et al.*, 2004). The up-regulation of an individual gene may therefore not confer a significant advantage to any one stress. Given the results from this chapter and with reference to cassava PPD, an improved strategy would be to over-express a combination of antioxidant genes at once. Such an approach has been used successfully to increase the stress-resistance of plants previously (Tang *et al.*, 2006; Lee *et al.*, 2007). Alternatively, a simpler method might be to over-express an oxidative stress master switch gene, such as a gene encoding an ROS-sensitive transcription factor, in root tissue and thereby modulate the expression of all the genes involved in responding to oxidative stress (Bhatnagar-Mathur *et al.*, 2007). Potential oxidative stress master switch genes have been isolated from plants, such as *DREB1B* and *SIPK* from rice (Gutha & Reddy, 2008; Cho *et al.*, 2009). An additional improvement would be to express a master switch

gene under the control of an oxidative stress-sensitive promoter, which would ensure the correct timing of expression and avoid wasting metabolic resources in synthesising transgenic protein unnecessarily (Bhatnagar-Mathur *et al.*, 2007). Unfortunately, to date such a promoter has not been tested in cassava storage tissue.

6 INVESTIGATING PPD IN CASSAVA

6.1 Introduction

6.1.1 Tools for researching PPD in cassava

Compared to the majority of other crop species and the model plant *Arabidopsis*, cassava has been the subject of little research. This is no doubt due to the lack of importance of this crop to Western food production and economics. As a result of this, a limited number of tools are available for cassava research. The cassava genome sequence has yet to be determined, although efforts to achieve this are currently ongoing. Similarly, a commercially available microarray chip is under development.

While the cassava genome sequence project is not yet complete, cDNA sequences for several genes proposed to be involved in PPD are known including *ASCORBATE PEROXIDASE* (APX), *CATALASE* (CAT), *SUPEROXIDE DISMUTASE* (SOD) and *PHENYLALANINE AMMONIA-LYASE* (PAL) (Lee *et al.*, 1999; Reilly, 2001; Reilly *et al.*, 2001; Reilly *et al.*, 2004; Shin *et al.*, 2005). Expression driven by the constitutive CaMV35S promoter is low in storage roots compared to leaf tissue (Zhang *et al.*, 2003), and so studies have been carried out to isolate storage root-specific promoters. Several promoters that confer such an expression pattern have been identified, like those of the *Mec-c15*, *Mec-c54* and *MecPAL2* cassava genes (Beeching *et al.*, 2000; Zhang *et al.*, 2003), as well as those of the class I *PATATIN* genes of potato. Promoters that are up-regulated in response to PPD in storage roots, such as *MecHNL4* and *MecPAL2*, may also prove useful (Hughes *et al.*, 1998; Beeching *et al.*, 2000; Reilly *et al.*, unpublished). In addition, cDNA libraries constructed from mRNA expressed post-harvest are available for screening, while robust protocols for the extraction of RNA and DNA also provide a useful resource for cassava researchers. Furthermore, a vast germplasm stock consisting of approximately 20,000 accessions is maintained at centres worldwide (Ng & Ng, 2002), which includes varieties of cassava with varying degrees of natural resistance to PPD.

6.1.2 Modulating the oxidative stress response through biotechnology

The goal of this research is to extend the shelf-life of cassava storage roots by modulating the PPD response. PPD is a process initiated and sustained by a prolonged oxidative burst that begins on harvesting. The strategy designed to modulate PPD is centred on increasing the ability of storage root tissue to scavenge reactive oxygen species (ROS) through a biotechnological approach. Genes encoding the ROS-scavenging enzymes – APX, CAT and SOD – are to be over-expressed in storage root tissue by driving their expression with the root-specific *StPAT* promoter.

The approach described above intended for application in cassava was first applied to the model plant *Arabidopsis* (chapter 5). The expression plasmids were able to confer root-specific transgene expression and subsequently were shown to increase antioxidant enzyme activity in root tissue compared to wild-type. In selected transgenic lines this increase was significant, although it did not appear to confer a substantial degree of resistance to oxidative stress when compared to wild-type plants. A subsequent experiment demonstrated that the APX activity of wild-type roots increased during exposure to H₂O₂-induced oxidative stress. In addition, previous reports suggested that the activities of both APX and SOD are post-transcriptionally induced by stress (Rizhsky *et al.*, 2004; Sunkar *et al.*, 2006). While useful in establishing the viability of the expression plasmids, the ability of *Arabidopsis* to respond efficiently to oxidative stress meant it was of limited use as a model for studying PPD since the phenomenon seems to arise in cassava storage roots due to their inefficiency in responding to oxidative stress. Indeed, PPD in cassava has been likened to a conventional plant wound response without efficient wound repair pathways or down-regulation of stress signalling (Reilly *et al.*, 2004). Over-expression of the three target genes in cassava storage roots therefore remained a viable strategy for modulating PPD and so cassava transformations were carried out to achieve this.

6.1.3 Natural variation facilitates research into the association between antioxidant enzymes and PPD

Natural variation within cassava permits the investigation of the potential influence of antioxidant enzymes on the oxidative stress response. Within the germplasm collection are varieties that range from PPD-susceptible to PPD-resistant, providing a valuable resource for investigating PPD in this manner. In addition, it

has been previously demonstrated that the magnitude and/or timing of antioxidant enzyme activity after harvesting in cassava is not sufficient to efficiently scavenge the ROS produced (Reilly *et al.*, 2001; Isamah *et al.*, 2003). Studies of this nature have utilised various cassava accessions with a range of susceptibilities to PPD, as well as differing techniques to extract protein from storage roots and a lack of consistency concerning cassava growth conditions. Therefore, the assessment of APX, CAT and SOD activities in PPD-susceptible and PPD-resistant varieties using consistent protocols should yield reliable data concerning the potential contribution of antioxidant enzymes to ROS scavenging after harvest.

6.1.4 Research aims

The primary aim of this chapter was to generate independent transgenic cassava lines over-expressing *MecAPX2*, *MecCAT1* and *MecSOD2* in storage root tissue in order to modulate PPD. An *Agrobacterium*-mediated method was used to transform cassava friable embryogenic calli (FEC) with the expression plasmids created in chapter 3 and used previously in chapter 5. Positive transformation lines were selected with the ultimate goal of screening these lines for susceptibility to PPD. The reporter expression plasmid should also be used to transform cassava in order to confirm the *StPAT* promoter is conferring storage root-specific transgene expression.

A second experiment aimed to establish the change in activity of APX, CAT and SOD in response to harvesting in cassava varieties with varying degrees of PPD susceptibility to determine if these enzymes were important in conferring tolerance to deterioration. Storage roots were harvested from available cassava varieties and assessed for their susceptibility to PPD, with reference to information from the literature. The storage root antioxidant enzyme levels were then assessed for the selected varieties.

6.2 Results – Generation of transgenic cassava

Cassava has traditionally proved recalcitrant to genetic transformation. This section describes the efforts of our laboratory to generate transgenic cassava plants and highlights the major modifications made to conventional transformation protocols. The transformation procedures outlined here were attempted first with the original pCAMBIA 1305.1 expression plasmid, which contained the 35S::GusP reporter cassette. This enabled simple screening of regenerated embryos/cotyledons via histochemical staining for GusP to identify positive transformants. All cassava transformation steps and analysis of potentially transgenic lines was performed by Simon Bull.

6.2.1 Generation of FEC tissue

The generation of FEC tissue was achieved via somatic embryogenesis. The first step in achieving this was to culture stem cuttings at 28 °C on growth media containing the synthetic cytokinin analogue BAP in the absence of light to induce bud formation. The formation of embryonic tissue was then induced by transferring the explants to growth media containing the synthetic auxin analogue picloram and again incubating at 28 °C in the dark. Embryonic material was sub-cultured every two weeks until FECs became visible (Figure 6.1), which usually occurred after approximately 10-12 weeks. Sub-culturing then continued with FEC material incubated in the light at 28 °C. After several cycles of sub-culturing, the FECs were suitable for *Agrobacterium*-mediated transformation.

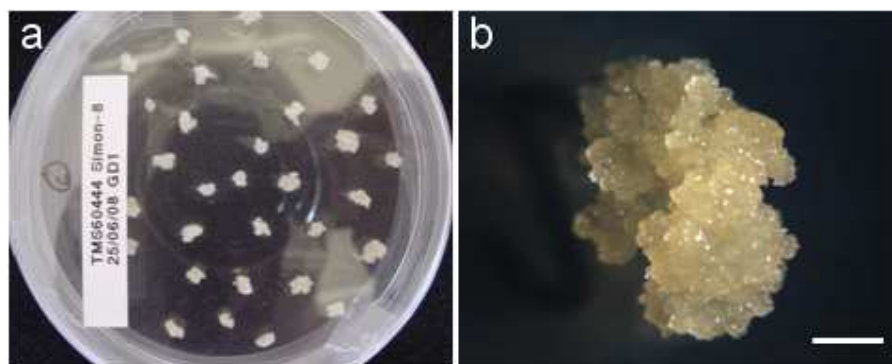


Figure 6.1: Maintenance of transformable friable embryogenic callus (FEC) tissue.
(a) FECs were maintained on sterile GD media in 9 cm Petri dishes at 28 °C in the light.
(b) Close up image of a FEC cluster, bar = 3 mm.

Throughout the sub-culture phase of FEC production it was observed that the FEC material had a wet appearance. It became apparent that the media setting agent used initially (Gelrite™) was responsible for this phenomenon and so it was replaced with a setting agent with no impurities (Noble agar).

6.2.2 Transformation of FEC tissue

Traditionally, cassava transformation protocols have dictated that after the generation of FEC tissue, FECs should be cultured in liquid proliferation media before and after inoculation with *Agrobacterium*. Trials using this method demonstrated that the structure of FECs changed during liquid culture, becoming more globular in appearance. In addition: FECs of a high quality were unable to survive for long in liquid culture; losses of 70% were experienced due to bacterial and fungal contamination; and the liquid culture steps were time-consuming, taking approximately 5 weeks.

Consequently, a plate-based method of FEC transformation was designed. *A. tumefaciens* culture harbouring the desired expression plasmid was dropped onto a FEC cluster and the two were co-cultivated for 3 days at 22 °C. The FECs were washed with liquid growth media and then transferred to sterile 100 µm mesh. This mesh was incubated on plates containing GD growth media supplemented with 250 µg/ml carbenicillin at 28 °C for 3-4 days to kill any remaining *A. tumefaciens* cells. After this time, the mesh holding the FECs was moved to GD media plates with 250 µg/ml carbenicillin and 5 µg/ml hygromycin. After incubation at 28 °C for 1 week, the mesh was moved to equivalent plates with the hygromycin concentration increased to 8 µg/ml. This was repeated 1 week later, increasing the hygromycin concentration to 15 µg/ml. The use of mesh to move the FECs between culture plates helped prevent mechanical damage to the FECs and maintained consistent growth conditions.

The gradual increase in hygromycin concentration in the growth media after FEC transformation appeared to help the FECs mature after co-cultivation with *A. tumefaciens*. In addition, a maximum hygromycin concentration of 15 µg/ml was used since the recommended concentration of 25 µg/ml was observed to kill all FECs. The lower concentration maintained sufficient selection pressure to prevent an abundance of false positives. Furthermore, the quality of hygromycin from several suppliers was inconsistent, which led to problems during screening for

positive transformants. Hygromycin supplied by Carl Roth proved to be consistently of a high grade and so was used for all hygromycin selection steps.

6.2.3 Regeneration of transformed cassava plants and confirmation of potential transgenic lines

The mesh harbouring the FECs was transferred to sterile MSN media, which contained the synthetic auxin analogue NAA, supplemented with 250 µg/ml carbenicillin and 15 µg/ml hygromycin. FECs were incubated at 28 °C until embryo/cotyledonous tissue began to develop (Figure 6.2a). After transformation with reporter expression cassettes, a sample of embryos/cotyledons was screened for positive transformants using a non-destructive GusP histochemical stain to determine if each specific transformation event had been successful (Figure 6.2b). Hygromycin-resistant embryos/cotyledons were transferred to growth media supplemented with BAP to encourage the growth of shoots, and also containing 100 µg/ml carbenicillin to kill any remaining *A. tumefaciens* cells. Shoots were then transferred to CBM to permit further growth and the development of roots.

In order to confirm lines as transgenic, plants derived from hygromycin-resistant embryos/cotyledons were subjected to three tests. The first test involved transplanting the growing tip of plants into CBM media containing 10 µg/ml hygromycin since successfully transformed plants have resistance to this selection agent. Shoot tips that had developed roots within 14 days of transplantation (Figure 6.2c) were considered to have successfully passed the first test. The second test employed PCR to genotype gDNA extracted from leaves of potential transgenic lines. Two PCRs were performed on each gDNA sample; one that amplified a region of the hygromycin resistance gene, and one that amplified a fragment that included *StPAT* promoter and target gene sequence (Figure 6.3a). Plants whose gDNA tested positive in both PCRs were considered to have successfully passed the second test (Figure 6.3b). The third test consisted of Southern blot analysis of gDNA. As for the gDNA PCR genotyping, the presence of both the hygromycin resistance gene and the target gene was verified for each sample, in this case using independent probes. Plants whose gDNA was shown to contain both the hygromycin resistance gene and the target gene through Southern analysis were considered to have passed the third test and assumed to be transgenic.

To date two *MecCAT1* and six *MecSOD2* transgenic lines have passed the rooting test, the gDNA PCR genotyping test and the Southern analysis test (Table 6.1). Meanwhile, fifteen lines transformed with *MecAPX2* have rooted in hygromycin and are awaiting gDNA PCR genotyping and Southern analysis. FECs transformed with the reporter expression cassette (*StPAT::GusP*) are currently on MSN media awaiting the formation of embryos/cotyledons. The transformation process is being repeated for all constructs to ensure sufficient transgenic lines are generated.

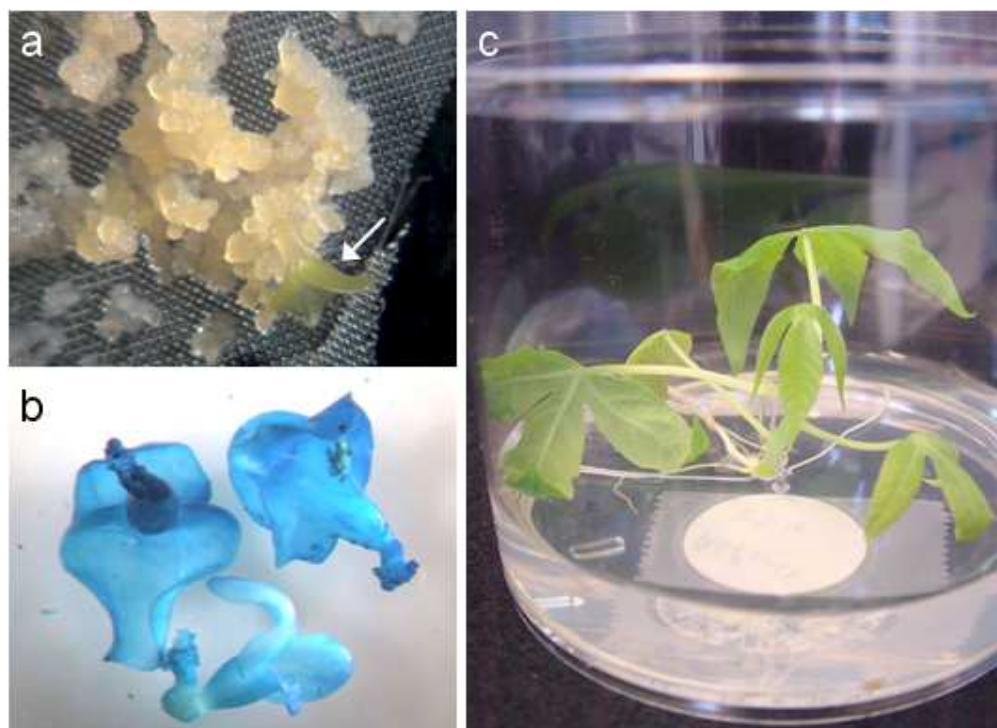


Figure 6.2: Regeneration of transformed cassava plants. (a) Cotyledonous tissue (white arrow) developing from transformed FEC tissue on mesh placed on MSN media containing 15 µg/ml hygromycin. (b) CaMV35S-driven GusP activity in large cotyledons derived from FEC tissue transformed with the pCAMBIA 1305.1 expression plasmid. (c) Regenerated transgenic cassava plant growing in CBM media with 10 µg/ml hygromycin.

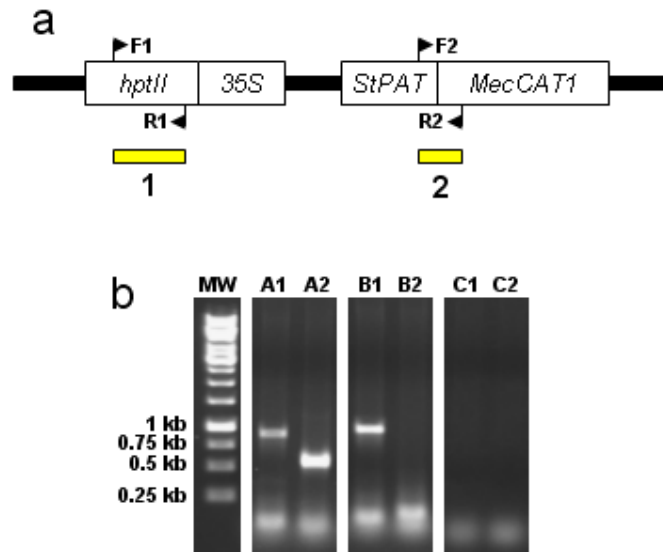


Figure 6.3: Analysis of potentially transgenic *MecCAT1* cassava lines via PCR genotyping. (a) Diagrammatic representation of the position of the two primers pairs in the *MecCAT1* expression cassette, yellow boxes represent amplicons 1 and 2, *hptII* = hygromycin resistance gene. (b) Results of gDNA genotyping – sample A was positive for both PCRs, sample B was positive only for PCR 1 and sample C was negative for both PCRs (false positive). MW = molecular weight marker.

Table 6.1: Progress – production of transgenic cassava plants

Cassette	FECs inoculated with <i>Agrobacterium</i> ?	Lines rooted in hygromycin	Lines positive – Southern analysis
<i>StPAT::MecAPX2</i>	Y	15	ND
<i>StPAT::MecCAT1</i>	Y	30	2
<i>StPAT::MecSOD2</i>	Y	24	6
<i>StPAT::GusP</i>	Y	ND	ND

ND = not determined

6.3 Results – Natural variation and antioxidant enzymes

6.3.1 Identification of cassava accessions showing natural variation concerning PPD resistance

The identification of cassava accessions with varying levels of PPD resistance started with a literature search. This resulted in the classification of seventeen cassava varieties into three groups: high PPD resistance (CW 429-1, CM 7033-3, CMC 2177-2, MBRA 337, MDOM 5, MCOL 2279, TMS 30572 and MVEN-77), intermediate PPD resistance (MNGA-1 and MNGA-2) and low PPD resistance (MCOL 22, SM 985-9, TMS 60444, MCOL 1505, HMC 1, CM 523-7, MPER 183 and MTAI 8) (Buschmann *et al.*, 2000; Reilly *et al.*, 2001; CIAT, 2006; Reilly *et al.*, 2007). A variety from each group was selected, based on availability in the University of Bath glasshouse. These were MVEN-77, MNGA-2 and TMS 60444.

6.3.2 Confirmation of PPD resistance levels

The varieties selected above were allowed to deteriorate to confirm their levels of PPD resistance. Storage roots of MVEN-77, MNGA-2 and TMS 60444 were harvested from mature plants. Five slices approximately 1 cm thick were cut from each storage root and placed in a ventilated Petri dish containing moist filter paper and incubated in sterile conditions at room temperature to permit deterioration to occur. Root slices showing signs of microbial decay were discarded. At 0, 6, 24, 48 and 72 hours after harvesting, the root slices were photographed and the extent of PPD was assessed via the traditional method of analysing the discolouration of storage root tissue.

After assessment of the photographs by eye (Figure 6.4), it was concluded that PPD progressed fastest in storage roots of TMS 60444. In this variety, discolouration could be detected 24 hours after harvest in the cortical parenchyma, which subsequently spread into the storage parenchyma. Storage roots incubated for 24, 48 and 72 hours were all soft to the touch, but showed no signs of microbial infection. Discolouration was detectable in storage roots of both MNGA-2 and MVEN-77 24 hours after harvest, although this was often restricted to a small region of the cortical parenchyma. However, at 48 and 72 hours after harvest discolouration had not spread to the same extent as it had in TMS 60444 storage roots, and discolouration present was of a lower intensity. In addition, MNGA-2 and MVEN-77 storage root slices remained firm over the time-course.

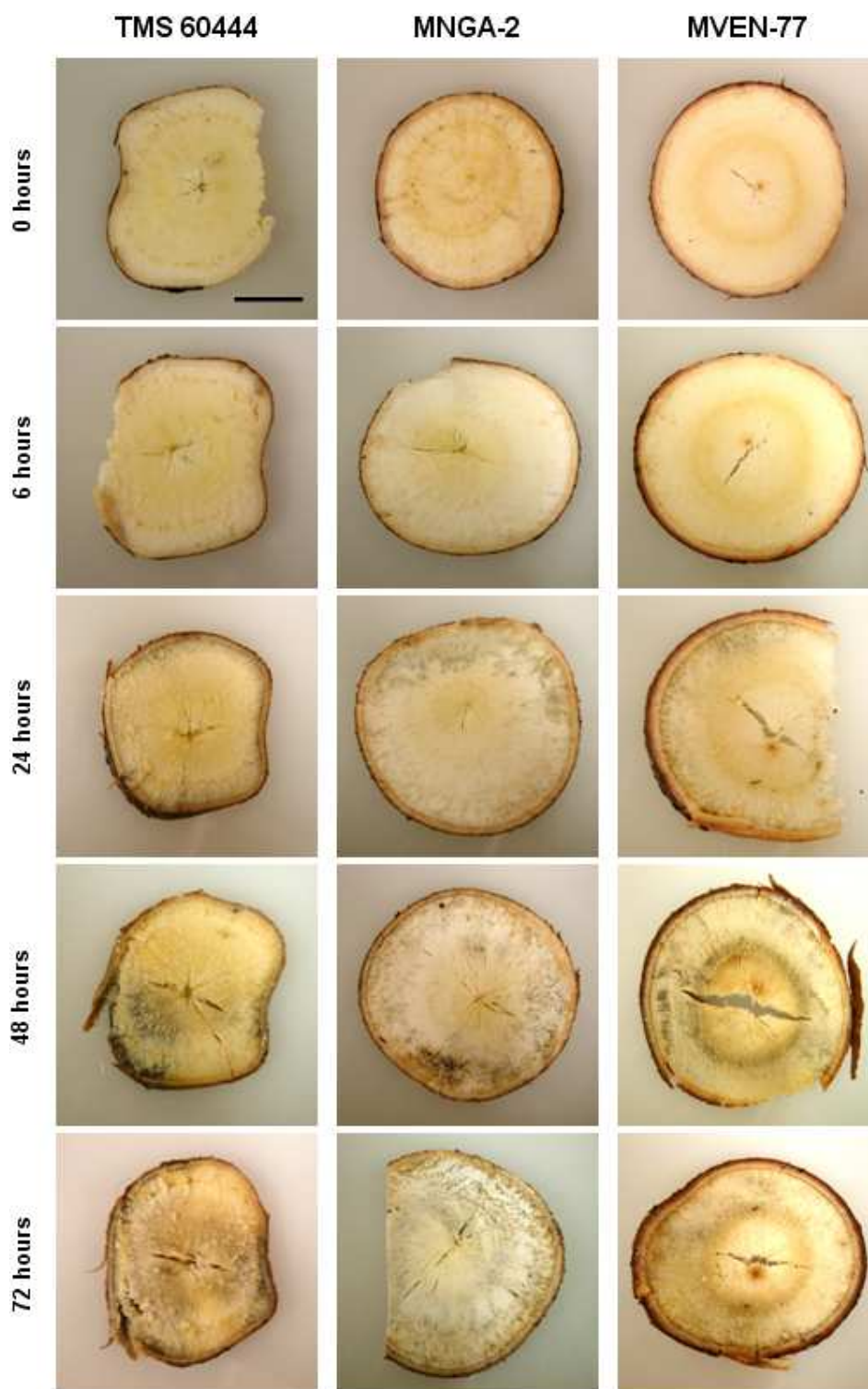


Figure 6.4: Cassava variety TMS 60444 is more susceptible to PPD than MNGA-2 or MVEN-77. Time after harvesting is given on the left hand side. Bar = 2 cm, all photographs taken at the same distance from storage root slices. Incomplete storage root slices are the result of removal of woody, non-storage root tissue prior to photographing. A representative root slice was photographed for each variety.

The extent of discolouration in the storage root slices shown in Figure 6.4 was then quantified. The area of discolouration of each root slice was measured using ImageJ and this was then converted to a percentage of the total storage root slice area.

The data from this analysis (Figure 6.5) confirmed that described above. Storage roots of variety TMS 60444 showed the highest percentage of discolouration at each time point. Interestingly, MVEN-77 (high PPD resistance) showed higher discolouration than MNGA-2 (intermediate PPD resistance) at 48 and 72 hours post-harvest suggesting that PPD proceeded faster in MVEN-77 under the conditions used here. However, only one storage root slice was measured here and therefore, given the high degree of variation observed within accessions and even between different storage roots of the same plant, should only be used as an indication. That said, the general pattern displayed in Figure 6.4 was observed consistently. In all subsequent analyses, the original PPD resistance classifications are used.

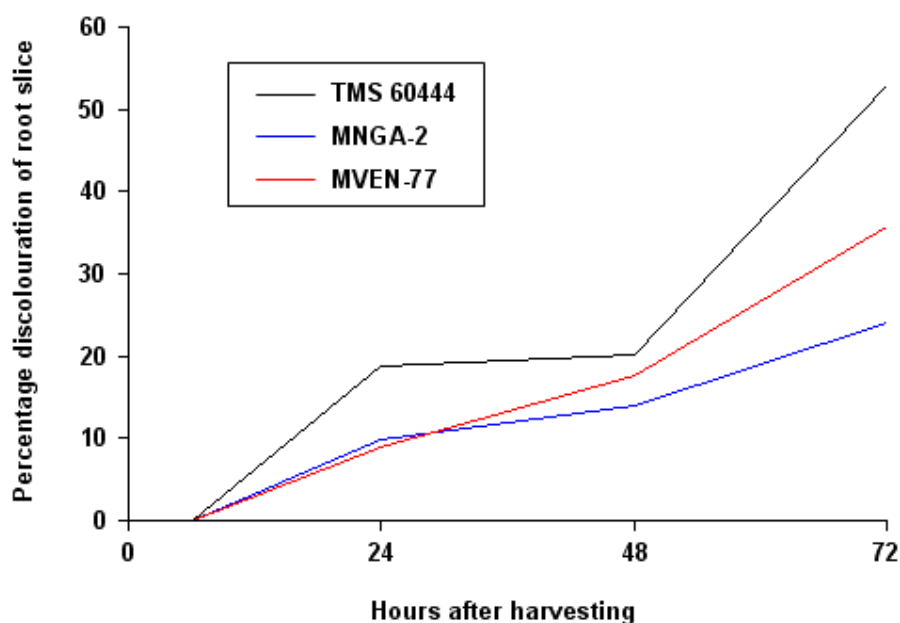


Figure 6.5: Storage root discolouration spread faster in TMS 60444 than MNGA-2 or MVEN-77. Percentage discolouration was measured for each root slice shown in Figure 6.X at 0, 6, 24, 48 and 72 hours post-harvest, n = 1.

6.3.3 Storage root APX activity does not correlate with PPD resistance

The APX activity of storage root tissue was measured at 0, 6, 24, 48 and 72 hours after harvesting for TMS 60444, MNGA-2 and MVEN-77. Two biological replicates were performed for each data point.

The APX activity of TMS 60444 (low PPD resistance) storage roots began to increase 24 hours after harvesting, with substantial increases seen at 48 and 72 hours post-harvest (Figure 6.6). The APX activity of MNGA-2 (intermediate PPD resistance) and MVEN-77 (high PPD resistance) also increased after 24 hours, although the degree to which they did so was reduced. For example, the APX activity of TMS 60444 storage roots 72 hours after harvesting was 2.07-fold higher than MNGA-2 and 2.93-fold higher than MVEN-77. These data suggested that APX was not an essential component of an efficient response to PPD. Importantly in all three varieties tested, the level of APX activity had not substantially increased until 48 hours after harvesting, by which time the visual symptoms of PPD were well established.

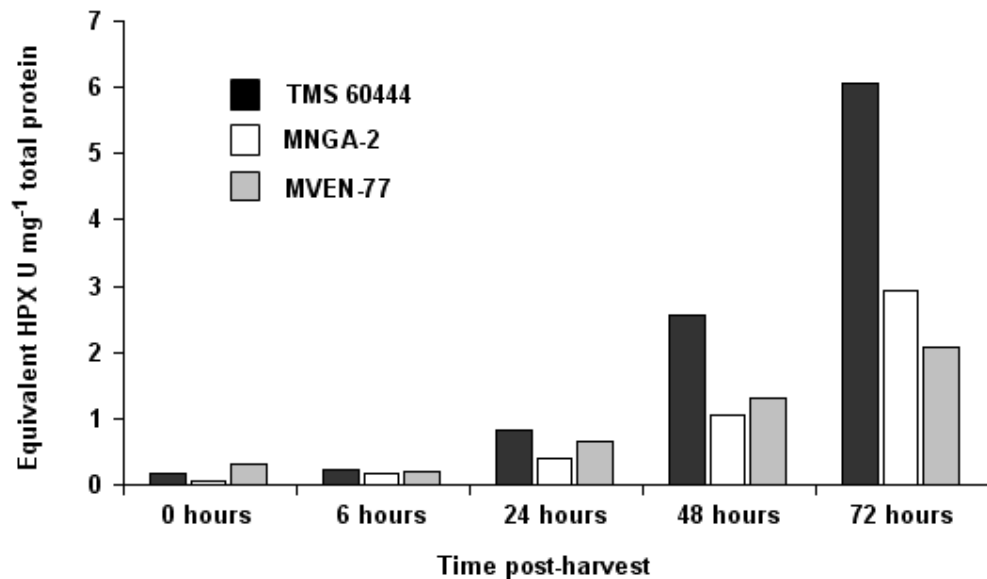


Figure 6.6: A PPD-susceptible variety of cassava shows greater up-regulation of APX activity in storage roots after harvest than two more PPD-resistant varieties. Mean HPX equivalent units of APX activity before and after harvest. TMS 60444 (low PPD resistance), MNGA-2 (intermediate PPD resistance), MVEN-77 (high PPD resistance), n = 2.

6.3.4 Storage root CAT activity may contribute to PPD resistance in variety MNGA-2

The above experiment was repeated, assaying for CAT instead of APX. The CAT activity of MNGA-2 storage roots was at a high level pre-harvest and was substantially up-regulated 6 hours after harvesting (Figure 6.7). Subsequently it returned to a pre-harvest level, although it remained higher than the CAT activity of the other varieties at all time points. The CAT activity of MVEN-77 storage roots was comparatively low pre-harvest, but up-regulation was observed until 24 hours post-harvest. The CAT activity of TMS 60444 storage roots remained stable throughout the time-course. It is possible that the high pre-harvest CAT activity of MNGA-2 storage roots, as well as the rapid up-regulation of activity after 6 hours, conferred some tolerance to the oxidative burst initiated upon wounding and so delayed PPD.

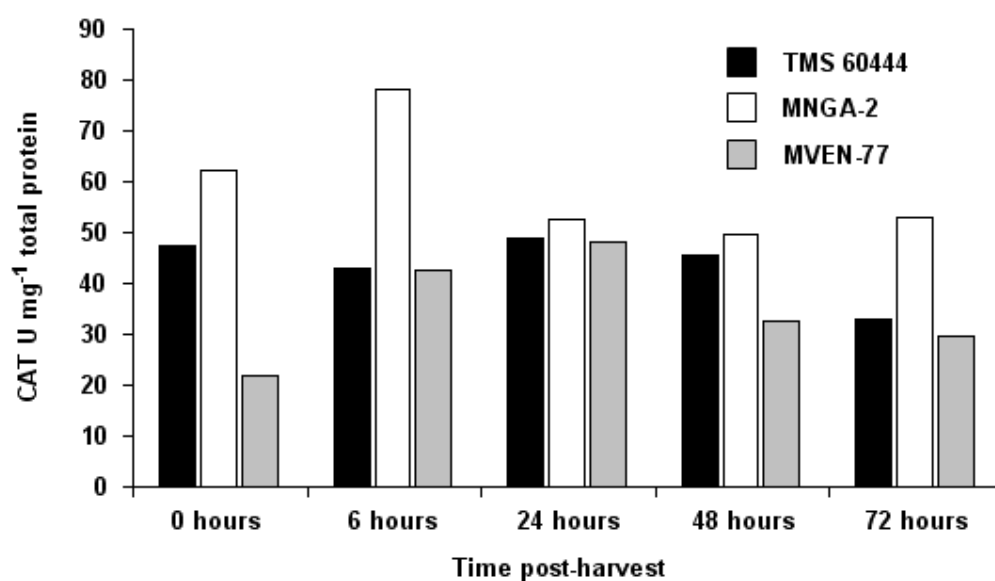


Figure 6.7: Storage root CAT activity is up-regulated immediately after harvesting in two more PPD-resistant varieties. Mean CAT activity before and after harvest. TMS 60444 (low PPD resistance), MNGA-2 (intermediate PPD resistance), MVEN-77 (high PPD resistance), n = 2.

6.3.5 Storage root SOD activity may contribute to PPD resistance in variety MNGA-2

The SOD activity of the storage root samples was then assessed. Before harvesting, variety MVEN-77 had the highest activity, although this was extensively down-regulated after just 6 hours (Figure 6.8). After this time, the SOD activity of this variety increased although pre-harvest levels were not attained. MNGA-2 storage root tissue demonstrated substantial up-regulation of SOD activity until 48 hours, while the SOD activity of TMS 60444 had declined 6 hours after harvesting but remained at a pre-harvest level at all other time points. This data indicates that in variety MNGA-2, the activity of SOD may be an important factor for delaying PPD.

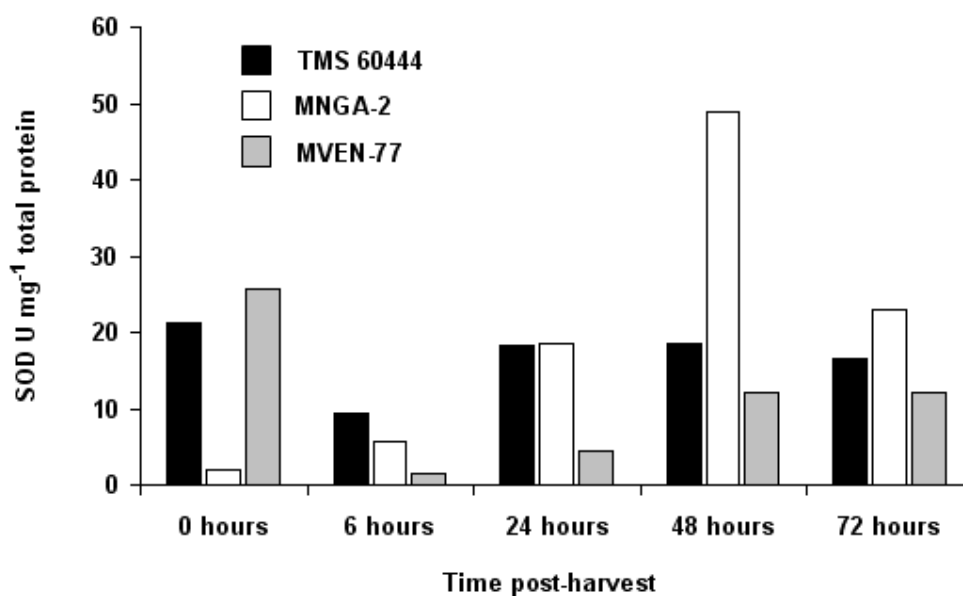


Figure 6.8: Storage root SOD activity is up-regulated after harvesting in MNGA-2. Mean SOD activity before and after harvest. TMS 60444 (low PPD resistance), MNGA-2 (intermediate PPD resistance), MVEN-77 (high PPD resistance), n = 2.

6.4 Discussion

6.4.1 Production of transgenic cassava

Cassava has traditionally been recalcitrant to genetic transformation. To date, just four genetic engineering studies in cassava have been published (Chellappan *et al.*, 2004; Siritunga *et al.*, 2004; Zhang *et al.*, 2005; Ihemere *et al.*, 2006). Attempts to transform cassava by this laboratory demonstrated that faithful replication of existing protocols was unable to successfully generate transgenic cassava plants. Through trial and error, it was possible to trouble-shoot key steps in the transformation process, and the improved protocols are now being used to successfully produce transgenic cassava plants – two lines over-expressing *MecCAT1* and six lines over-expressing *MecSOD2* have been generated.

Trouble-shooting of the existing protocols highlighted key factors to improve transformation efficiency. The major difference in the protocol used here compared to existing protocols was the absence of a liquid proliferation phase for FECs before and after inoculation with *Agrobacterium*. In addition, the concentration of hygromycin for selection of transgenics was at most 15 µg/ml; the recommended concentration of 25 µg/ml proved too high to permit FEC survival. Other important factors that affected transformation efficiency were the quality of the media setting agent and hygromycin. The trouble-shooting process has enabled the development of a more robust protocol, which is essential in preparing this technology for transfer to African research centres where it is needed most.

6.4.2 The association between antioxidant enzyme activity and PPD resistance

Three varieties of cassava were selected based on their varying levels of resistance to PPD, as described in the literature. Assessment of the progression of PPD here confirmed that TMS 60444 had the least resistance to PPD, while MNGA-2 and MVEN-77 showed similar levels of resistance. It was previously reported that MNGA-2 had an intermediate level of resistance to PPD and that MVEN-77 had high resistance (Buschmann *et al.*, 2000). The reason for the conflicting data here could be due to the conditions under which the storage root slices were allowed to deteriorate. In addition, the methods of quantifying PPD here and in the previous studies were similar but not identical, and the care with which roots were harvested was impossible to standardise and can have a large influence on the speed of deterioration. Furthermore, a great deal of variation

exists within cassava varieties and as such a larger sample size is required to definitively classify MNGA-2 and MVEN-77 as having either intermediate or high resistance to PPD.

Since the process of transforming cassava is difficult and time-consuming, published data concerning the over-expression or knock-down of genes in cassava is extremely rare. As a result, the importance of APX, CAT and SOD in reference to PPD is not clear as inferences have only be made from native expression levels and enzyme activities post-harvest. Furthermore, these inferences have been made based on data from a range of cassava accessions that show variation in their resistance to PPD. However the conclusions drawn from such investigations, along with the PPD resistance of the varieties used, are given below.

A microarray comparing CMC 2177-2 (high PPD resistance) storage roots pre- and post-harvest showed that one cassava APX isoform, *MecAPX2*, was transiently up-regulated at 12 and 24 hours post-harvest, after which it returned to pre-harvest levels (Reilly *et al.*, 2007). While the APX activity of storage roots has not been assessed in response to harvesting, the total peroxidase (POX) activity has been measured. The POX activity of Oyolu (unknown PPD resistance) storage roots increased slightly after 24 hours and then declined, remaining below the pre-harvest level (Isamah, 2004).

The two known cassava *CAT* genes are differentially regulated in response to harvesting according to microarray data (CMC 2177-2, high PPD resistance). *MecCAT2* was up-regulated after 24 and 72 hours, with expression remaining approximately at the pre-harvest level after 12 and 48 hours, while *MecCAT1* expression was transiently increased at 48 hours post-harvest (Reilly *et al.*, 2007). Analysis of *MecCAT1* expression in MCOL22 (low PPD resistance) by Northern blot suggested up-regulation of *MecCAT1* did not occur until 96 hours after harvesting, inferring a positive correlation between *CAT* expression and PPD resistance (Reilly *et al.*, 2001; Reilly *et al.*, 2004). Localisation of CAT activity supported an association between CAT and PPD by demonstrating that TMS 30572 (high PPD resistance) storage roots maintained a high level of activity for six days after harvesting, while the activity in MCOL22 (low PPD resistance) storage roots decreased dramatically four days after harvesting and remained low (Reilly *et al.*, 2001).

MecSOD1 expression was induced by wounding, high temperature and chemical stressing agents in cassava leaf tissue (Lee *et al.*, 1999), with *MecSOD2*

similarly up-regulated in response to stresses (cassava varieties not given for either study) (Shin *et al.*, 2005). Surprisingly then, the expression of both *MecSOD1* and *MecSOD2* was shown not to be modulated after harvesting by a microarray analysis (CMC 2177-2, high PPD resistance) (Reilly *et al.*, 2007). The situation is further complicated by SOD activity data (cassava variety not given), which indicated that activity increased slightly after 24 hours before declining considerably over the subsequent 72 hours (Isamah *et al.*, 2003).

The only logical conclusion that could be drawn from the above is that the antioxidant enzyme response of cassava storage roots was, in general, not sufficient in timing or magnitude to prevent PPD, which had usually commenced irreversibly by 24 hours post-harvest. However, the analysis of three cassava accessions with varying degrees of PPD resistance in this chapter suggested that the antioxidant enzyme response of selected accessions may be sufficient to confer some resistance to PPD. Since the varieties used here and in the above studies differed, direct comparisons should not be sought.

Of the varieties tested here, MNGA-2 showed the lowest percentage discolouration of storage root tissue 48 and 72 hours post-harvest, and also the greatest up-regulation of antioxidant activity post-harvest. The level of active SOD protein increased for the first 48 hours after harvest, while the CAT activity of MNGA-2 storage roots was up-regulated after 6 hours but subsequently declined. CAT is considered to be involved in mass detoxification of H_2O_2 during stress rather than fine-tuning of H_2O_2 levels, at least in other plant species (Mittler, 2002). PPD is considered to be a ROS-mediated process and so rapid up-regulation of CAT after harvesting could play an important role in delaying the process. APX activity was also up-regulated at each successive time point after harvesting, although not substantially until 48 hours after harvesting.

Storage roots of TMS 60444 showed the highest percentage discolouration at all time points, supporting published information describing them as having low PPD resistance (Buschmann *et al.*, 2000). Unsurprisingly then, neither CAT nor SOD activities were up-regulated post-harvest in this variety. TMS 60444 storage roots did show the largest up-regulation of APX activity, although again this was not substantial until 48 hours post-harvest, by which time the symptoms of PPD were well established. The role of APX in fine-tuning, rather than large-scale scavenging of H_2O_2 suggests APX may not be implicated in conferring PPD resistance in any case (Mittler, 2002).

The resistance of MVEN-77 storage roots to PPD was unlikely to be a consequence of antioxidant enzyme activity. The APX activity increased at each successive time point after harvesting, but again this was only substantial 48 hours post-harvest. The SOD activity was down-regulated approximately 18-fold 6 hours post-harvest and remained lower than the pre-harvest level throughout, while the CAT activity was up-regulated between 0 and 24 hours after harvesting but never surpassed the CAT activity of MNGA-2 at any time-point.

An inappropriate antioxidant response by TMS 60444 may explain its low resistance to PPD. In comparison, the antioxidant response of MNGA-2 could confer some of the PPD resistance shown by this variety. However, it is likely that the resistance of MVEN-77, and MNGA-2 to an extent, is conferred by agents other than the three antioxidant enzymes studied here. A major contributor to this may be carotenoids, antioxidant molecules that can react with virtually any radical species to form more stable products (Krinsky & Yeum, 2003). In plant systems they have been implicated in the quenching of singlet oxygen ($^1\text{O}_2$) produced during photosynthesis and preventing lipid peroxidation chain reactions (Havaux & Niyogi, 1999; Smirnoff, 2005). Carotenoids have been shown to be associated with PPD in cassava as the level of carotenoids tends to decline as PPD progresses (Gloria & Uritani, 1984). In addition, cassava accessions vary in their carotenoid content and a positive correlation was found between carotenoid content and resistance to PPD (Sánchez *et al.*, 2006). It is possible that high levels of carotenoids are implicated in conferring PPD resistance to storage roots of MVEN-77 plants. Unfortunately, white fleshed cassava storage roots that are low in carotenoids are often preferred by consumers and starch producers. These varieties are therefore more susceptible to PPD and have a lower nutritional value (Sánchez *et al.*, 2006).

In conclusion, the PPD resistance of any particular variety is most likely a combined result of the antioxidant enzyme activity, carotenoid content and pre-harvest factors such as pruning and growth conditions (Tanaka *et al.*, 1984; van Oirschot *et al.*, 2000). However, in the case of MNGA-2, antioxidant enzymes may play a considerable role in conferring PPD resistance. What is also clear is that the antioxidant enzyme response of TMS 60444 (low PPD resistance) in response to harvest-induced wounding is insufficient in timing and/or magnitude to prevent PPD. Care should be taken during the analysis of enzyme activities and other markers of PPD, since variation within cassava varieties is high due to

environmental and genetic influences (Rodríguez, 2001). Storage root over-expression of APX, CAT and SOD remains a viable strategy for increasing resistance to PPD, since the period immediately after harvest is important and activities of the enzymes should be high at the time of harvesting and thereafter.

6.4.3 Further work

Concerning the generation of transgenic cassava material, the transformation process is on-going for the three target gene expression cassettes and the *StPAT::GusP* reporter expression cassette. After the identification of transgenic reporter lines, histochemical localisation of GusP should be performed on small *in vitro* grown plants to confirm that *StPAT* promoter-driven *GusP* expression is largely root-specific in cassava. This procedure must be repeated on storage roots when they have developed. In addition, GusP localisation assays on wounded cassava leaves from transgenic reporter lines should be carried out to determine if the *StPAT* promoter is wound-responsive in cassava.

Target gene over-expression lines must be analysed for transgene expression levels in root tissue and subsequently root antioxidant enzyme activity relative to untransformed TMS 60444 (wild-type) plants. Lines with significant increases in root enzyme activity compared to wild-type plants should be grown to maturity in the University of Bath glasshouse. It is essential that once identified transgenic plants are sufficiently multiplied during their *in vitro* growth phase to ensure a good supply of storage roots in the glasshouse since losses may occur through invertebrate attack or over-watering. Storage roots should be assessed for their resistance to PPD using a quantitative scoring method.

Concerning the natural variation experiment, due to the limited availability of storage roots in the University of Bath glasshouse only two biological replicates of the antioxidant enzyme assays were performed for each cassava variety. Assessment of the enzyme activities of more biological replicates would allow statistical analysis of the data and provide more reliable results. In addition, a more robust and qualitative analysis of the extent of PPD should be incorporated. Ideally this would be based on the percentage discolouration of the storage roots, as described above, and also the percentage fluorescence and firmness of the storage roots. Furthermore, to improve the confidence with which associations between PPD resistance and antioxidant enzyme activity can be drawn, a larger

number of cassava varieties should be analysed. The concurrent determination of the carotenoid content using a previously described method (Sánchez *et al.*, 2006) would also provide informative data concerning the basis of PPD resistance.

7 DISCUSSION

The aim of this investigation was to design and evaluate a strategy for extending the shelf-life of cassava storage roots. The strategy used was centred on increasing the ability of storage roots to scavenge ROS by modulating the expression of genes encoding antioxidant enzymes. Root-specific target gene over-expression was achieved using the *StPAT* promoter, which was shown to be rapidly induced on wounding in non-root tissue of *Arabidopsis* plants. Target gene over-expression in the model *Arabidopsis* was able to increase antioxidant enzyme activity but did not confer increased oxidative stress resistance. However, the oxidative stress response of cassava roots is poor in comparison to *Arabidopsis* and so implementation into cassava remains a viable strategy. The antioxidant response of MNGA-2 storage roots is good and may contribute to the natural PPD resistance seen in this cassava variety.

7.1 The *StPAT* promoter is wound-inducible

The *StPAT* promoter version used in this investigation was shown to be wound-inducible. *StPAT* promoter-derived expression was seen as soon as two minutes after wounding, and was unaffected by MeJa, ethylene or ROS. In addition, none of the abiotic stresses tested here was able to induce *StPAT* promoter activity. It is likely that, given the lipolytic acyl hydrolase (LAH) activity of PATATIN and the rapid nature of the response, that it acts early during a wound-response to free fatty acids from cell membranes. These fatty acids may subsequently be involved in oxylipin (for example, jasmonate) biosynthesis or they may be toxic to microbial invaders. β -1,3 glucanase activity of PATATIN also indicates a role in fungal defence. Further work is needed to fully characterise the wound-inducibility of the *StPAT* promoter and the *cis* regulatory motifs responsible for conferring this activity. It is interesting to note that the *StB33 PATATIN* promoter was not wound-inducible, suggesting diverse functions for the PATATIN proteins. Of the nine *Arabidopsis* patatin-like proteins (PLPs), only *AtPLP2* and *AtPLP7* demonstrated pathogen-responsiveness supporting the hypothesis that PATATIN family members have diverse roles (La Camera *et al.*, 2005).

Wound-inducible expression conferred by the *StPAT* promoter should have little impact on storage root deterioration, since root *GusP* activity appears unaltered in *Arabidopsis* reporter lines in response to wounding. However, the *StPAT* promoter could be employed in transgenic studies to address a range of other constraints to cassava production. For example, cassava mosaic disease (CMD) is spread by whitefly (*Bemisia tabaci*) and severely affects cassava production – in 2002, approximately 20-25% of African cassava was lost to CMD (Storey & Nichols, 1938; Zhang *et al.*, 2005). Once identified, the *cis* regulatory motif responsible for rapid *StPAT* wound-inducibility could be fused to a leaf-specific promoter. Over-expression of a gene encoding a protein toxic to whitefly under the control of the fusion promoter should prevent the whitefly feeding on the cassava leaves and thereby help restrict the spread of CMD since whitefly must puncture (and therefore wound) cassava leaves during feeding. The minimum time required for transmission of CMD from whitefly to cassava has been reported as being ten minutes (Dubern, 1994), longer than the two minutes it takes the promoter to be induced. In addition, the *StPAT* promoter could be used to improve the current strategies for detoxification of cyanogenic glycosides. Transgenic cassava over-expressing *HYDROXYNITRILE LYASE* (*HNL*) have been generated to increase the turnover of the substrate of HNL, the harmful acetone cyanohydrin (Siritunga *et al.*, 2004). The constitutive CaMV35S promoter was employed to drive transgene expression. However acetone cyanohydrin is only produced upon mechanical tissue disruption and therefore constitutive expression of *HNL* wastes metabolic resources. Substitution of the CaMV35S promoter for the *StPAT* promoter would ensure *HNL* is only expressed in the desired tissue.

7.2 An antioxidant approach to solving PPD

Expression cassettes consisting of genes encoding antioxidant enzymes under the control of the *StPAT* promoter conferred an increase in root-specific antioxidant enzyme activity in all *Arabidopsis* lines tested compared to wild-type. Importantly, a consistent increase in antioxidant enzyme activity did not occur in leaf tissue, confirming the results seen in Chapter 4 that indicated largely root-specific expression conferred by the promoter. The suitability of the *StPAT* promoter for driving transgene expression in cassava may be limited due to its wound-inducibility, since valuable resources will be utilised in producing antioxidant proteins unnecessarily in non-storage root tissue should wounding occur.

Increases in antioxidant enzyme activity up to 3.68-fold wild-type levels were unable to confer considerable resistance to oxidative stress on the roots of transgenic *Arabidopsis* plants compared to wild-type. As highlighted in Chapter 5, this is likely to be an effect of the efficient nature with which *Arabidopsis* can respond to oxidative stress. Studies involving root-specific antioxidant transgene over-expression are rare. However, previous studies concerning the constitutive over-expression of genes encoding antioxidant enzymes in *Arabidopsis* have yielded similar increases in enzyme activity but much greater increases in oxidative stress resistance (Murgia *et al.*, 2004; Sarowar *et al.*, 2005). This may be a result of the constitutive nature of transgene expression in these studies, enhancing the antioxidant status of the whole plant and not just the root system. While suitable for studies in *Arabidopsis*, the CaMV35S promoter is unsuitable for driving transgene expression in cassava since expression driven by this promoter is low in storage roots (Zhang *et al.*, 2003). In addition, it is advantageous to restrict transgene expression to the desired region of the plant since constitutive expression wastes resources and can result in abnormal development (Bhatnagar-Mathur *et al.*, 2007).

Ultimately, it is unimportant that an increase in antioxidant enzyme activity did not confer oxidative stress resistance on *Arabidopsis* roots. The use of the model species *Arabidopsis* was chiefly a method of demonstrating the efficacy of the expression plasmids. In cassava storage roots, a markedly poor antioxidant response contributes considerably to the PPD process. Here it was observed that a PPD-susceptible variety of cassava showed no increase in CAT or SOD activity after harvesting. The response of plant cells to moderate oxidative stress is usually to increase ROS scavenging in order to reduce oxidative damage. In contrast, when plant cells encounter high levels of oxidative stress, ROS scavenging is often suppressed in order to bring about ROS-induced PCD and contain the stress source (Mittler *et al.*, 1999; Apel & Hirt, 2004; Gao *et al.*, 2008). It is possible that a poor post-harvest antioxidant response causes the over-accumulation of ROS, which triggers PCD in cassava storage roots (Figure 7.1). If this is the case, over-expression of genes encoding ROS-scavenging enzymes should still provide a degree of resistance to PPD.

Given the nature of the antioxidant response of cassava storage roots to harvesting, there is not an appropriate model species in which to study PPD in cassava. However, the significant increase in root antioxidant enzyme activity seen in selected transgenic *Arabidopsis* lines suggests the expression plasmids

generated here have the potential to modulate PPD in cassava storage roots, whether the mode of action of PPD is ROS-induced PCD or ROS-derived oxidative damage. That said, a concern for the suitability of this approach for delaying PPD results from the influence of cyanide on antioxidant enzyme activity. When H_2O_2 accumulates to high levels in plant cells, CAT is largely responsible for scavenging this ROS. However, CAT is sensitive to cyanide-inhibition (Ogura & Yamazaki, 1983) and so over-expression of *MecCAT1* in storage roots may not confer an increase in CAT activity due to cyanogenesis initiated on harvesting (Figure 7.1). Cyanide-inhibition has also been demonstrated for SOD (Ozaki *et al.*, 1988).

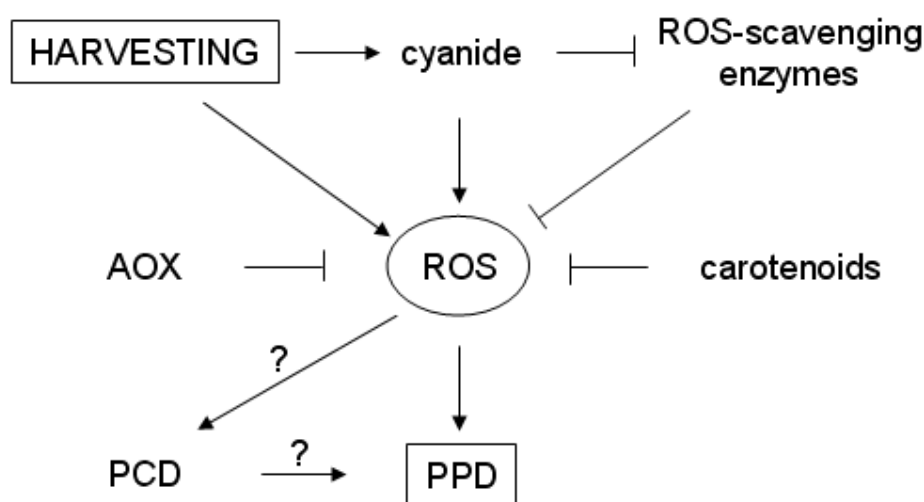


Figure 7.1: A model of the interaction of factors that potentially modulate PPD in cassava storage roots. AOX = alternative oxidase, ROS = reactive oxygen species, PCD = programmed cell death, PPD = post-harvest physiological deterioration. Arrows with accompanying question marks indicate unconfirmed interactions.

7.3 Future directions for cassava PPD research

Antioxidants are undoubtedly important in the physiological responses of cassava storage roots to wounding incurred on harvesting. However, other potential contributors such as programmed cell death (PCD) and cyanogenesis might provide opportunities for developing alternative strategies for modulating PPD (Figure 7.1).

ROS may act as signalling molecules during PPD to bring about PCD since accumulation of ROS and the suppression of ROS scavenging appear to be essential for the progression of PCD in plants (Mittler *et al.*, 1999; Apel & Hirt, 2004; Gao *et al.*, 2008). A proof-of-concept investigation is underway in this laboratory, involving storage root over-expression of human *BCL2* and *BCL-XL*, and *CED9* from *Caenorhabditis elegans*. These genes encode anti-apoptotic proteins, thereby suppressing cell death in animal systems (Dickman *et al.*, 2001; Williams & Dickman, 2008). Over-expression of *BCL-XL* and *CED9* has previously been shown to confer resistance to MV-induced photo-oxidative stress on tobacco plants (Mitsuhara *et al.*, 1999), suggesting that the products of these genes are able to inhibit ROS-induced cell death (Hockenbery *et al.*, 1993). Furthermore, these plants also demonstrated resistance to low temperature and salt stress (Qiao *et al.*, 2002) inferring that abiotic stress resistance can also be enhanced through the over-expression of genes encoding anti-apoptotic proteins.

It is possible that cyanogenic compounds released on wounding interfere with the METC and increase ROS production, or inhibit antioxidant enzyme activity and thereby contribute to PPD. 'Sweet' cassava varieties containing low levels of cyanogenic glycosides may be grown but are more susceptible to herbivore attack (McMahon *et al.*, 1995). Therefore, a future strategy might seek to increase cyanogen turnover post-harvest through biotechnology. Transgenic cassava plants over-expressing *HNL* under the control of a double CaMV35S promoter have been generated (Siritunga *et al.*, 2004). *HNL* breaks down acetone cyanohydrin to yield acetone and free cyanide, which is then extracted with water or left to volatilise (Siritunga & Sayre, 2004). The transgenic plants showed an increased turnover of acetone cyanohydrin, but maintained wild-type levels of linamarin pre-harvest and so retained their defences against herbivores. Unfortunately, the transgenic storage roots generated in this study were not assessed for their resistance to PPD. Another potential approach is to over-express a gene encoding ALTERNATIVE OXIDASE (AOX) in cassava storage roots, since AOX is able to reduce ROS production and functions through a cyanide-resistant pathway (Maxwell *et al.*, 1999; Juszczuk & Rychter, 2003). The generation and analysis of transgenic plants over-expressing *A. thaliana* AOX is currently in progress as part of the BioCassava Plus project. It should be noted that a BioCassava Plus project aiming to increase the stable carotenoid content of cassava storage roots through a biotechnological approach is also underway.

Molecular research in cassava is set for a rapid expansion in the near future. The arrival of the annotated cassava genome sequence and a commercially-available cassava microarray chip will allow more intensive investigations into PPD. For example, screening of the genome sequence will permit the identification of genes with sequence homology to stress master-switch genes previously isolated in other plant species. Modulation of the expression of such genes could prove a successful strategy in delaying PPD. In addition, a microarray comparing gene expression in PPD-resistant and PPD-sensitive varieties pre- and post-harvest would highlight genes important in conferring PPD resistance. The promoters of genes that were up-regulated on harvesting could be isolated and employed as stress- or wounding-sensitive promoters in over-expression studies. However, for the cassava genome sequence and microarray chip to have a substantial impact on cassava research, a robust, reliable and efficient protocol for the genetic transformation of this species must first be formulated. This would also allow cassava research to be carried out in countries that grow the crop, to ensure real problems are being addressed (Masona *et al.*, 2001).

A biotechnological approach focussing on preventing ROS accumulation post-harvest maintains the potential to extend the shelf-life of cassava storage roots.

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9 APPENDIX

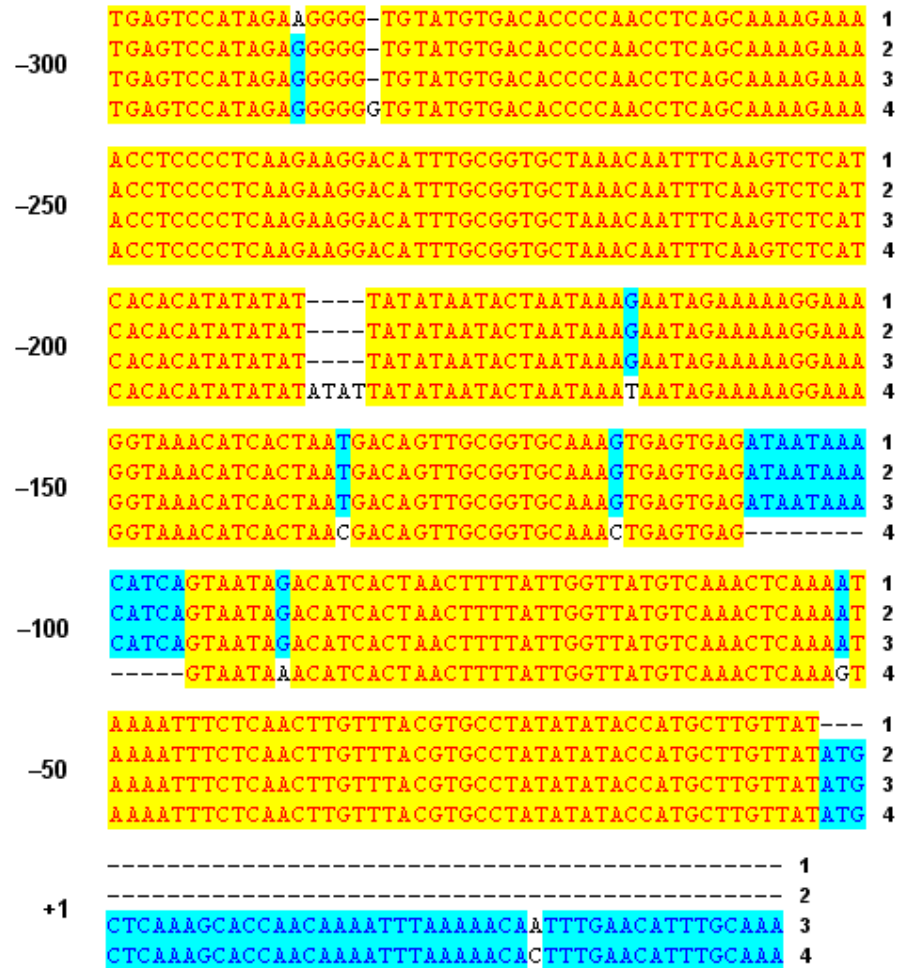


Figure 9.1: Sequence alignment of four class I *PATATIN* promoters. An unpublished *PATATIN* promoter sequence, AY485645 (1), the *StPAT* promoter (2), a Maris Piper *PATATIN* promoter, X03956 (Bevan *et al.*, 1986) (3) and the *StB33* promoter, X14483 (Rocha-Sosa *et al.*, 1989) (4). Sequences aligned with Vector NTI 10. Numbers on the left hand side refer to the base pair position relative to the 3' terminus of the *StPAT* promoter.

Cited in Chapter 4, page 76.

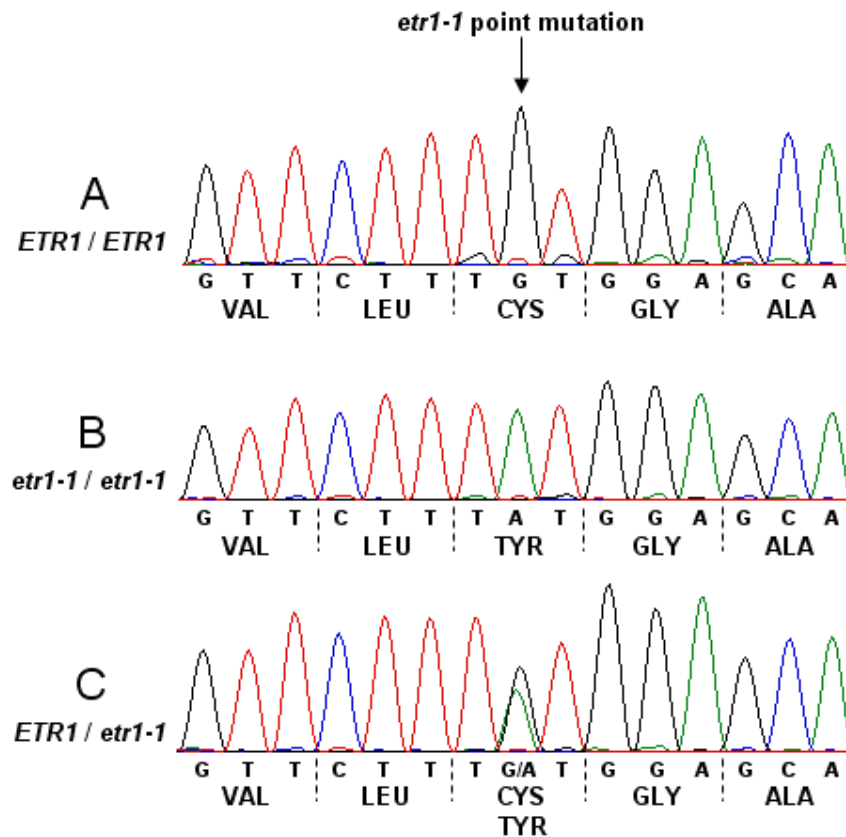


Figure 9.2: Sequence analysis of the parent lines wild-type Col-0 and *etr1-1*, and their F₁ progeny. Sequence chromatograms displaying base pairs 187-201 of the *ETR1* coding sequence. The *etr1-1* point mutation (base pair 194) is indicated with an arrow. Sequencing was carried out for parent lines (A) wild-type, Col-0 (*ETR1 / ETR1*) and (B) homozygous *etr1-1* mutants (*etr1-1 / etr1-1*), as well as their F₁ progeny, (C) heterozygous *etr1-1* mutants (*ETR1 / etr1-1*).

Cited in Chapter 4, page 89.

Table 9.1: Putative *cis* regulatory motifs in the *StPAT* promoter – unrelated to wounding.

Motif Function	Motif Name	Motif Sequence	Hits
Anaerobic fermentation	Anaero1	AAACAAA	2
	Anaero3	TCATCAC	2
ARR-1 binding element	ARR1 box	NGATT	14
Auxin/salicylic acid responsiveness	ASF1	TGACG	5
	NDE element	CATATG	1
Common plant promoter element	CAAT	CAAT	7
	GATA box	GATA	10
	RRE	CANNTG	6
	TATA box	(variations of) TATA	25
	BIHD1Os	TGTCA	7
Disease resistance	BIHD1Os	TGTCA	7
Embryo/endosperm specificity	-300 element	TGHAAARK	1
	AACA	AACAAAC	1
	DPBFcore	ACACNNG	2
	GCN4	TGTGTCA	2
	Skn1	GTCAT	4
	MBSII	AAAAGTTAGTTA	1
Flavanoid biosynthesis	HEXTa	ACGTCA	1
Histone gene regulation	YACT	YACT	17
Mesophyll specificity	NOD1	AAAGAT	2
Nodule specificity	NOD2	CTCTT	2
	-10PEHVPSBD	TATTCT	3
Plastid gene expression	Box II	ATAGAA	2
	S1F box	ATGGTA	1
	GTGA(Nt)	GTGA	17
Pollen specificity	Pollen1	AGAAA	6
	CPB element	TATTAG	3
Protein binding (general)	DOFcore	AAAG	19
	EEC	GANTTNC	2
	HD-Zip 3	GTAAT(G/C)ATTAC	1
	RHE	KCACGW	1
Root specificity	ROOT	ATATT	5
	MARA Box	AATAAAYAAA	2
Scaffold attachment region	ACGT	ACGT	4
Stress responsiveness (general)	CBF	RYCGAC	1
	CCA1	AAMAATCT	2
	GT-1	GRWAAW	11

H = not G. K = G/T. M = A/C. N = any nucleotide. R = A/G. W = A/T. Y = C/T

Bold = also found in *SPORAMIN* promoter sequence

Cited in Chapter 4, pages 92 & 93.